Lentiviral Vector Mediated Haematopoietic Stem Cell Gene Therapy For Mucopolysaccharidosis Type IIIA

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Abstract

Lentiviral Vector Mediated Haematopoietic Stem Cell Gene Therapy For Mucopolysaccharidosis Type IIIA

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Mucopolysaccharidosis type III (Sanfilippo) is comprised of four phenotypically similar lysosomal storage disorders (MPS IIIA-D) caused by the deficiency of enzymes that catabolise heparan sulphate (HS). Progressive accumulation of HS results in abnormal behaviour, progressive cognitive and motor impairment and death in mid-teens. There are currently no treatments for MPS III.

To assess the effect of novel therapeutics in the mouse models of MPS III it is necessary to examine the effect on primary storage of HS, secondary storage and behaviour. The reported behaviour of MPS IIIA and B mice is conflicting therefore we developed a one-hour open field test, performed at the same time of day during a period of hyperactivity observed in a previous circadian rhythm study of MPS IIIB mice. At 8 months of age MPS IIIB mice were hyperactive, with increased rapid exploratory behaviour and a reduction in immobility time. The MPS IIIA mice presented with the same behavioural phenotype as the MPS IIIB mice and were significantly hyperactive at 4 and 6 months of age and also displayed a reduced sense of danger. The hyperactivity and reduced sense of danger observed in the mice is consistent with the patient phenotype.

Whilst haematopoietic stem cell transplant (HSCT) is the standard therapy used to treat the similar HS storage disorder MPS I Hurler, it is ineffectual in MPS IIIA. We hypothesise that HSCT failure in MPS IIIA is due to insufficient enzyme production in the brain by donor-derived microglial cells. By increasing expression of N-sulphoglucosamine sulphohydrolase (SGSH) we may be able to treat MPS IIIA. Therefore we compared the effect of HSCT using normal haematopoietic stem cells (WT-HSCT) to lentiviral overexpression of SGSH in normal cells (LV-WT-HSCT) or MPS IIIA cells (LV-IIIA-HSCT) in MPS IIIA mice, using the behavioural tests developed.

SGSH activity in the brain of MPS IIIA recipients was not significantly increased by WT-HSCT, but was significantly increased by LV-IIIA-HSCT and LV-WT-HSCT. HS was significantly reduced by all transplants but the best treatment was LV-WT-HSCT. Neuroinflammation, indicated by the number of microglia in the brain, was significantly reduced by all treatments but remains significantly elevated. GM2 gangliosides were significantly reduced by WT-HSCT and LV-WT-HSCT and were no longer significantly elevated, but LV-IIIA-HSCT had no significant effect. Critically LV-WT-HSCT corrected the behaviour at 4 and 6 months of age whilst the other treatments had no significant effect.

LV-WT-HSCT and WT-HSCT reduced GM2 gangliosides and neuroinflammation equally but only LV-WT-HSCT corrected behaviour and primary HS storage, suggesting they are the important factors in MPS IIIA pathology.

LV-WT-HSCT corrects the neurological phenotype in MPS IIIA mice and is a clinically viable approach to treat MPS IIIA and other neuropathic lysosomal storage disorders.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright Statement

Alternative Format Thesis

This thesis has been presented in the alternative format. It therefore comprises an introduction and methods section as would be found in a normal thesis, followed by three chapters prepared as papers for submission to peer reviewed journals. There is then a final conclusion section to summarise the papers and thesis as a whole.

Chapter 3, “Hyperactive behaviour in the mouse model of Mucopolysaccharidosis IIIB in the open field and home cage environments” was accepted for publication in Genes, Brain and Behavior on 30th May 2011. In this paper the behavioural testing of the MPS IIIB mice was carried out by Marcelina Malinowska but the analysis and writing of the paper was carried out by Alex Langford-Smith.

Chapter 4, “Female Mucopolysaccharidosis IIIA mice exhibit hyperactivity and a reduced sense of danger in the open field test” was accepted for publication in PloS ONE on 9th September 2011. The behavioural testing and analysis of the MPS IIIA was performed by Alex Langford-Smith. Other authors provided technical assistance and/or critically appraised the manuscript.

Chapter 5, “Hematopoietic stem cell and gene therapy corrects primary neuropathology and behavior in Mucopolysaccharidosis IIIA mice where conventional transplant fails” was submitted on 10th October 2011 to Blood. The majority of the experiments in this paper were carried out by Alex Langford-Smith, except the analysis of HS by AMAC, which was performed by Dr Rebecca Baldwin, and cloning of SGSH into the lentiviral vector which was performed by Dr Fiona Wilkinson and Dr William Bennett.

Acknowledgement

I would like to acknowledge the help and support of my supervisors Dr Brian Bigger and Dr Fiona Wilkinson and everybody else in the lab. I would also like to thank the staff of the BSU for their assistance and experience.

None of this work could have been carried out without the funding of the Sanfilippo Children's Research Foundation, the UK and Irish Societies for Mucopolysaccharide Diseases and the Biotechnology and Biological Sciences Research Council.

Dedication

I would like to dedicate this thesis to my wife, Kia, and my Mum without whom I would not have started this and certainly would not have finished it.

Abbreviations

AAV – Adeno Associated Virus

Ad – Adenovirus

ANOVA - ANalysis Of VAriance

APC – Allophycocyanin

ARSA – Arylsulphatase A

BBB – Blood Bran Barrier

BMT – Bone Marrow Transplant

bp – Base Pairs

BSA – Bovine Serum Albumin

CMV – CyclomegaloVirus

CNS – Central Nervous System

cPPT – Central Polypurine Tract

CS – Chondroitin Sulphate

DAPI – 4',6-diamidino-2-phenylindole

DMEM - Dulbecco's Modified Eagle's Medium

DNA – Deoxyribonucleic acid

DS – Dermatan Sulphate

eGFP – Enhanced Green Fluorescent Protein

ERT – Enzyme Replacement Therapy

FACS – Fluorescence-activated cell sorting

FCS - Foetal Calf Serum

FITC – Fluorescein Isothiocyanate

GAG – Glycosaminoglycan

GAPDH – Glyceraldehyde 3-Phosphate Dehydrogenase

GlcA – Glucuronic Acid

GlcNAc – N-acetylglucosamine

GMP – Good Manufacturing Practice

GTAC – Gene Therapy Advisory Committee

HEK – Human Embryonic Kidney

HS - Heparan Sulphate

HSCT – Haematopoietic Stem Cell Transplant

IdoA – Iduronic Acid

IDUA – α –L-Iduronidase

IMS - Industrial Methylated Spirits

KS – Keratan Sulphate

LAMP2 – Lysosomal-Associated Membrane Protein 2

LB - Luria-Bertani

LSD - Lysosomal Storage Disorder

LTR – Long Terminal Repeat

LV – Lentiviral Vector

M-6-P – Mannose-6-Phosphate

MANOVA - Multivariate ANalysis Of VAriance

MHRA – Medicines and Healthcare products Regulatory Agency

MLD – Metachromatic Leukodystrophy

MOI – Multiplicity Of Infection

MPS – Mucopolysaccharide

MPS I – Mucopolysaccharidosis type I

MPS IIIA – Mucopolysaccharidosis type IIIA

MPS IIIB – Mucopolysaccharidosis type IIIB

MSD – Multiple Sulphatase Deficiency

NAGLU – α-N-Acetylglucosaminidase

NB-DGJ – N-Butyldeoxygalactonojirimycin

NB-DNJ – N-Butyldeoxynojirimycin (Miglustat)

NSAID – Nonsteroidal Anti-Inflammatory Drug

OMIM – Online Mendelian Inheritance in Man

PBMC – Peripheral Blood Mononuclear Cells

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PE – R-Phycoerythrin

PEG – Polyethylene glycol

PEI – Polyethylenimine

QPCR – Quantitative Polymerase Chain Reaction

RNA – Ribonucleic acid

RPMI – Roswell Park Memorial Institute

RRE – Rev Response Element

RT – Room Temperature

S/MAR – Scaffold/Matrix Attachment Region

SCF – Stem Cell Factor

SCID – Severe Combined Immunodeficiency

SCN – Suprachiasmatic Nucleus

SEM – Standard Error of the Mean

SFFV – Spleen Focus-Forming Virus Promoter

SGSH – N-Sulphoglucosamine Sulphohydrolase

SIN – Self Inactivating

SMGD – Steroid-Mediated Gene Delivery

SRT – Substrate Reduction Therapy

TNFα – Tumour Necrosis Factor α

VAMP2 – Vesicle-Associated Membrane Protein 2

VIP – Vasoactive Intestinal Peptide

VSVG – Vesicular Stomatitis Virus Glycoprotein

VTA – Ventral Tegmental Area

WAS – Wiskott Aldrich Syndrome

WPRE – Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element

WT – Wild Type

Treatment Abbreviations

WT – Untreated age matched wild type control mice

WT-HSCT – A transplant with wild type haematopoietic stem cells

LV-WT-HSCT – A transplant with wild type haematopoietic stem cells that have been transduced with SGSH lentiviral vector

LV-IIIA-HSCT – A transplant with MPS IIIA haematopoietic stem cells that have been transduced with SGSH lentiviral vector

MPS IIIA - Untreated age matched MPS IIIA control mice

Chapter 1 – Introduction

1.1 Mucopolysaccharide Diseases

1.1.1 Lysosomal Storage Disorders

Lysosomal storage disorders (LSD) are a family of over fifty genetic diseases that result in intra-lysosomal accumulation of undegraded macromolecules (Wraith 2002). These multisystem progressive disorders occur at a frequency of around 1 in 7,500 live births (Meikle *et al.* 1999; Poorthuis *et al.* 1999). The majority of LSDs are caused by inactivation of an enzyme involved in the degradation of substrates within the lysosome. For example, Mucopolysaccharidosis type I is caused by inactivity of the enzyme α-L-iduronidase and Mucopolysaccharidosis type IIIA by a lack of N-sulphoglucosamine sulphohydrolase (SGSH, sulphamidase) activity. However, proteins involved in lysosomal maintenance, enzyme activation, transporters and receptors are also causes of disease.

Lysosomal storage disorders can be grouped by primary storage material. Some LSDs are primarily lipid storage disorders; gangliosides are accumulated in GM1 gangliosidosis, Sandhoff and Tay-Sachs. Glucosylceramide and glucosylsphingosine are stored in Gaucher disease, sphingomyelin in the family of Niemann-Pick diseases and sulphatide in Metachromatic Leukodystrophy. Another group of LSDs accumulate glycogen, such as Pompe disease.

As the stored molecules differ in various LSDs the clinical symptoms vary, but they are characteristically progressive disorders due to gradual substrate accumulation. This usually results in a progressive loss of function of the affected tissue or tissues. The tissues affected vary with the stored substrate and the severity of disease.

1.1.2 Mucopolysaccharide Diseases

Mucopolysaccharide (MPS) diseases are a subset of lysosomal storage disorders that accumulate glycosaminoglycans (GAG). They have an incidence of approximately 1 in 26,000 live births in the UK (UK MPS Society). All the MPS diseases are caused by deficiencies in enzymes involved in GAG degradation, resulting in the accumulation of GAGs in the lysosome, cells and extracellular matrix. Different subtypes of MPS were originally classified by their clinical

phenotype but now specific enzyme deficiency and clinical severity are also considered (Table 1-1).

The symptoms and the severity of the diseases differ but all present with developmental problems.

MPS type I is an autosomal recessive disease caused by mutations in the IDUA gene coding for the enzyme α-L-iduronidase. The variation in severity within MPS I is so large that it is characterised as three separate diseases based on clinical observation; a severe form called MPS I Hurler (MPS IH), an intermediate form called MPS I Hurler/Scheie (MPS IH/S), and a mild form called MPS I Scheie (MPS IS). The latter two possess residual enzyme activity, whilst Hurler is usually the result of total enzyme inactivation by a stop or frameshift mutation. The life expectancy for MPS IH is 7.9 years, MPS IH/S patients survive until on average 21.6 years and MPS IS sufferers have a normal life expectancy (Moore *et al.* 2008). These variable phenotypes are due to the location and type of mutation in IDUA. Single amino acid changes in the catalytic domain of an enzyme can dramatically affect the activity of the enzyme, whereas changes in other regions of the enzyme may have little effect. MPS IH manifests with severe progressive neurodegeneration, organomegaly, dysostosis multiplex in the bones and joints and coarse facial features.

Morquio syndrome (MPS IVA and B) presents primarily with severe skeletal abnormalities and organomegaly caused by keratan sulphate (KS) storage. MPS VI and IX present with skeletal and/or joint problems and are associated with dermatan sulphate (DS) and chondroitin sulphate (CS) storage. MPS I, II and VII store dermatan sulphate (DS) (MPS VII stores CS as well) and present with skeletal abnormalities, however they also store heparan sulphate which appears to be associated with neurodegeneration. MPS III mainly stores heparan sulphate, resulting in a primarily neuronal phenotype.

MPS	Alternative name	Missing Enzyme	Major Phenotype	Gene	Stored GAG
MPS IH*	Hurler Syndrome	α-L-iduronidase	Neuronal/ skeletal	*Idua*	DS, HS
MPS IH/S*	Hurler-Scheie				
MPS IS*	Scheie Syndrome				
MPS IIA†	Hunter Syndrome	iduronate 2-sulphatase	Neuronal/ skeletal	*Ids*	DS, HS
MPS IIB†					
MPS IIIA	Sanfilippo Syndrome A	N-sulphoglucosamine sulphohydrolase	Neuronal	*Sgsh*	HS
MPS IIIB	Sanfilippo Syndrome B	α-N-acetylglucosaminidase	Neuronal	*Naglu*	HS
MPS IIIC	Sanfilippo Syndrome C	heparin-α-glucosaminide N-acetyltransferase	Neuronal	*Hgsnat*	HS
MPS IIID	Sanfilippo Syndrome D	N-acetylglucosamine-6-sulphatase	Neuronal	*Gns*	HS
MPS IVA	Morquio Syndrome A	galactosamine (N-acetyl)-6-sulphate sulphatase	Skeletal	*Galns*	KS
MPS IVB	Morquio Syndrome B	β-1-galactosidase	Skeletal	*Glb1*	KS
MPS VI	Maroteaux-Lamy Syndrome	arylsulphatase B	Skeletal	*Arsb*	CS, DS
MPS VII	Sly Syndrome	β-glucuronidase	Neuronal/ skeletal	*Gusb*	CS, DS, HS
MPS IX	Hyaluronidase Deficiency	hyaluronoglucosaminidase 1	Skeletal/ joints	*Hyal1*	CS, DS

Table 1-1 MPS family of diseases

* MPS IH is the severe form of MPS I, MPS IS is the mild form and MPS IH/S is an intermediate form.
† MPS IIA is the severe form of MPS II and MPS IIB is the mild form.

1.1.2.1 MPS III (Sanfilippo)

MPS III, or Sanfilippo disease contains four subtypes; A, B C and D. MPS IIIA is the most common in Northern Europe and IIIB in the Mediterranean regions (Emre *et al.* 2002). In the UK from 1990-2006 there have been 126 patients identified with MPS III with an incidence of 1:83,000 live births. Of those, 71% (89 patients) are MPS IIIA giving an incidence of 1:116,000 live births (Heron *et al.* 2011). Types A, B, C and D are deficient in different enzymes; N-sulphoglucosamine sulphohydrolase (SGSH), α-N-acetylglucosaminidase (NAGLU), heparin-α-glucosaminide N-acetyltransferase (HGSNAT) and N-acetylglucosamine-6-sulphatase, respectively. In all types of MPS III, HS is stored, and the different subtypes are clinically indistinguishable, although MPS IIIC usually has a slower progressive phenotype than MPS IIIA or B. The first symptoms of MPS III can manifest as early as 7 months (± 1.2 months), though diagnosis occurs on average at 4.6 years (± 2.6 years) (Meyer *et al.* 2007). The first symptoms are delayed developmental milestones and behavioural changes, which include sleep disturbances, hyperactivity, a reduced

sense of danger and aggressive behaviour (Bax *et al.* 1995; Fraser *et al.* 2002; Valstar *et al.* 2008; Malm *et al.* 2010; Wegrzyn *et al.* 2010). The majority (86-92%) of MPS IIIA patients have coarse facial features (Meyer *et al.* 2007; Heron *et al.* 2011). Other clinical symptoms include recurring diarrhoea and ear nose and throat infections (Sivakumur *et al.* 1999). As demonstrated in Table 1-2 and Table 1-3, development is initially relatively normal, then individuals begin to progressively lose their motor functions and cognitive abilities (Meyer *et al.* 2007; Heron *et al.* 2011). The maximum developmental age achieved is on average 3-4 years (Valstar *et al.* 2011). The clinical phenotype and progression of MPS IIIA and B are indistinguishable (see Table 1-3) and patients die on average at 15 years of age (Cleary *et al.* 1993; Meyer *et al.* 2007; Heron *et al.* 2011).

Speech Age ± SD /yrs	**Cognitive Function** Age ± SD /yrs	**Motor Function** Age ± SD /yrs
Impairment of speech **2.8 ± 1.9**	Deterioration of cognitive function **3.0 ± 1.4**	Clumsy walking **4.1 ± 3.6**
Speech difficult to understand **5.7 ± 2.7**	Loss of interest in environment **8.2 ± 3.7**	Aided walking **9.9 ± 4.3**
Loss of speech **8.2 ± 3.7**	Unresponsiveness **13.1 ± 4.2**	Wheel chair/immobile **12.4 ± 5.3**

Table 1-2 Clinical progression of MPS IIIA

This table demonstrates the deterioration of speech, cognitive and motor functions in a study of German MPS IIIA patients adapted from Meyer *et al.* 2007.

Measure Median/yrs ± SD	**MPS IIIA**	**MPS IIIB**
Independent walking	1.3 ± 0.4	1.1 ± 0.3
Two-word combination	3.0 ± 1.1	2.6 ± 2.6
Onset of cognitive delay	3.0 ± 1.4	3.5 ± 1.3
Abnormal behaviour	3.5 ± 3.3	4.0 ± 6.8
Loss of independent walking	10.2 ± 3.8	13.3 ± 9.4
Loss of relational interactions	10.0± 3.7	12.0± 3.5

Table 1-3 Clinical progression of MPS IIIA and IIIB

This table demonstrates the deterioration and similarity of French MPS IIIA and B patients, adapted from Heron *et al.* 2011.

1.2 Mucopolysaccharide Disease Pathology

1.2.1 Primary Storage

The primary storage material in all Sanfilippo diseases is heparan sulphate (HS), but how HS causes neurodegeneration is not known. HS has many important roles that may be dysregulated by changes in the sulphation pattern or amount of HS that may influence the disease phenotype.

HS is the primary storage material and can be measured directly using electrospray ionisation tandem mass spectroscopy (Mason *et al.* 2006), however this is an expensive technique and hard to optimise. The heparin cofactor II–thrombin complex is a biomarker that is elevated in serum from patients with MPS I, II, III, IV and VI (Randall *et al.* 2006; Randall *et al.* 2008; Clarke *et al.* 2011; Langford-Smith *et al.* 2011c). However while it is a good biomarker for those MPS diseases that store DS (e.g. MPS I), HS causes a smaller elevation in heparin cofactor II–thrombin complex levels so it is less effective in MPS III and is not significantly elevated in the MPS IIIA and B mouse models (Langford-Smith *et al.* 2010).

1.2.1.1 Heparan Sulphate Structure and Function

HS is a glycosaminoglycan (GAG) formed from repeating disaccharides, of glucuronic acid (GlcA) or iduronic acid (IdoA) linked to N-acetylglucosamine (GlcNAc) (Turnbull *et al.* 2010). HS comprises N-acetylated (NA) regions that have undergone limited modifications that consist mainly of GlcA-GlcNAc repeats. This basic repeat can then be modified. Highly sulphated S-domains are typically 3-8 disaccharides long and are modified by N- and O- sulphation. Epimerization of most glucuronic acid (GlcA) to iduronic acid (IdoA) also occurs. There are also NA/NS domains where alternating N-acetylation and N-sulphation occurs and a mix of GlcA and IdoA is present.

The range of modification of HS and their presence in the extracellular matrix and on the cell surface means that they can regulate protein-protein interactions by altering protein structures, increasing protein stability and limiting protein movement (Bishop *et al.* 2007; Turnbull *et al.* 2010). Often HS chains are linked to proteins to form proteoglycans, which facilitates these interactions. They have a

rapid turnover of 3-4 hours in cultured cells (Turnbull *et al.* 2010) and are responsive to extracellular signals from growth factors (Schmidt *et al.* 1995), therefore HS can act as a dynamic signal modification molecule (Turnbull *et al.* 2001). HS has a diverse range of roles and includes but is not limited to, interactions with fibroblast growth factor and receptor, transforming growth factors, interleukins, chemokines, extracellular matrix and enzymes (Bishop *et al.* 2007). The roles of HS are well reviewed in (Bishop *et al.* 2007).

GAG degradation is mediated by a series of enzyme reactions (Figure 1-1, Figure 1-2, Figure 1-3, Figure 1-4). The different enzyme deficiencies can result in storage of slightly different forms of HS with different end structures depending on which step is blocked. For example in MPS IH the terminal iduronic acid residue makes up a larger proportion of HS than in MPS III (Holley *et al.* 2011). Additionally in MPS IH the storage of HS results in the upregulation of glucosaminyl N-deacetylase/N-sulphotransferase (NDST) enzymes. These enzymes are responsible for defining the location of sulphated regions and upregulation of these enzymes increases the amount of *N*- and *O*- sulphation. Therefore the storage of HS can result in the accumulation of unusual HS end products and the increase in sulphation of HS, which could affect the function of HS and impact directly on disease pathology (Holley *et al.* 2011). Similar findings have also been observed in MPS IIIB (McCarty *et al.* 2011).

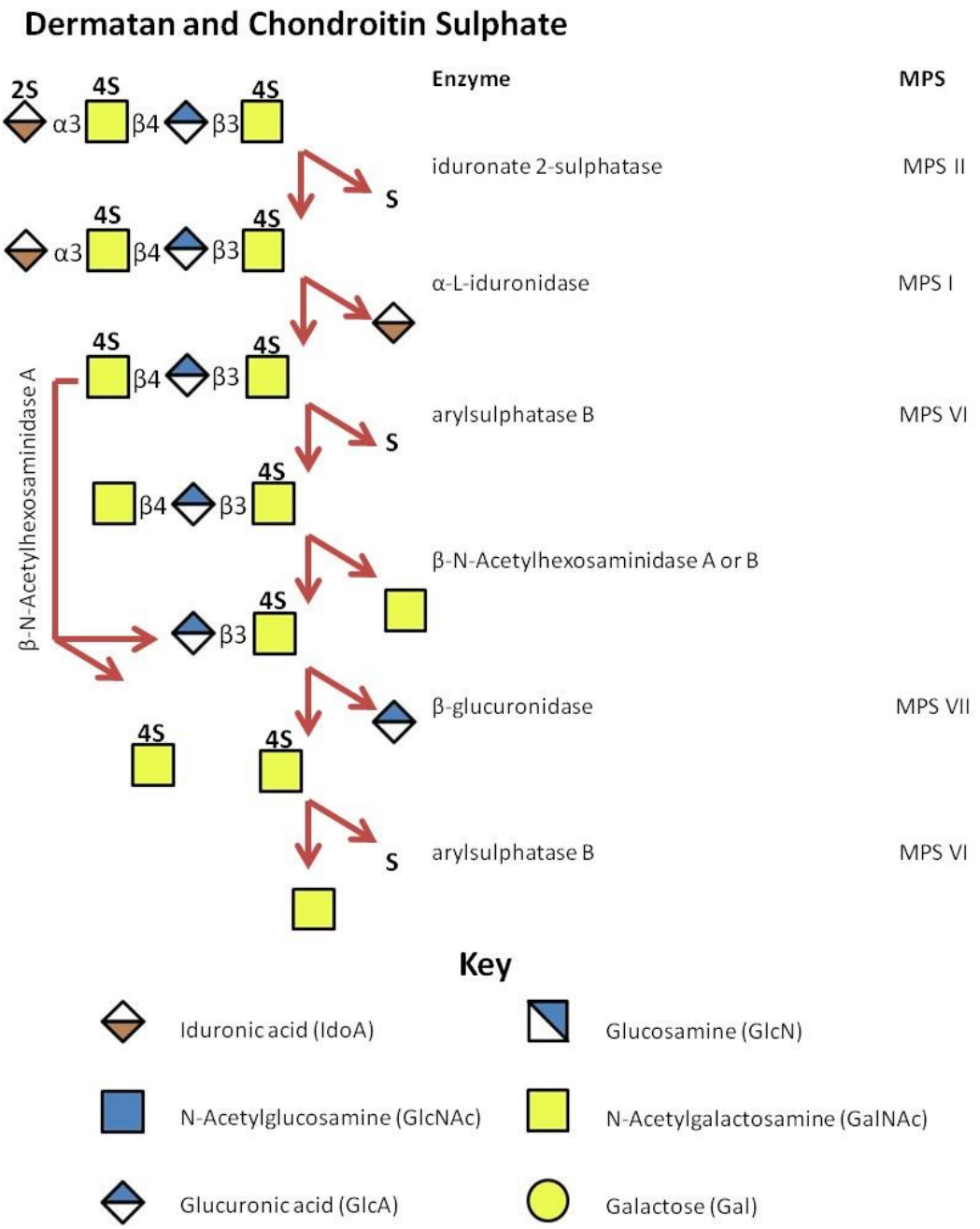


Figure 1-1 The pathway of dermatan and chondroitin sulphate degradation

This figure depicts the degradation of dermatan and chondroitin sulphate. The enzyme name and MPS disorder resulting from the deficiency of each enzyme are listed next to the relevant reaction. Adapted from Essentials of Glycobiology (Freeze 2009).

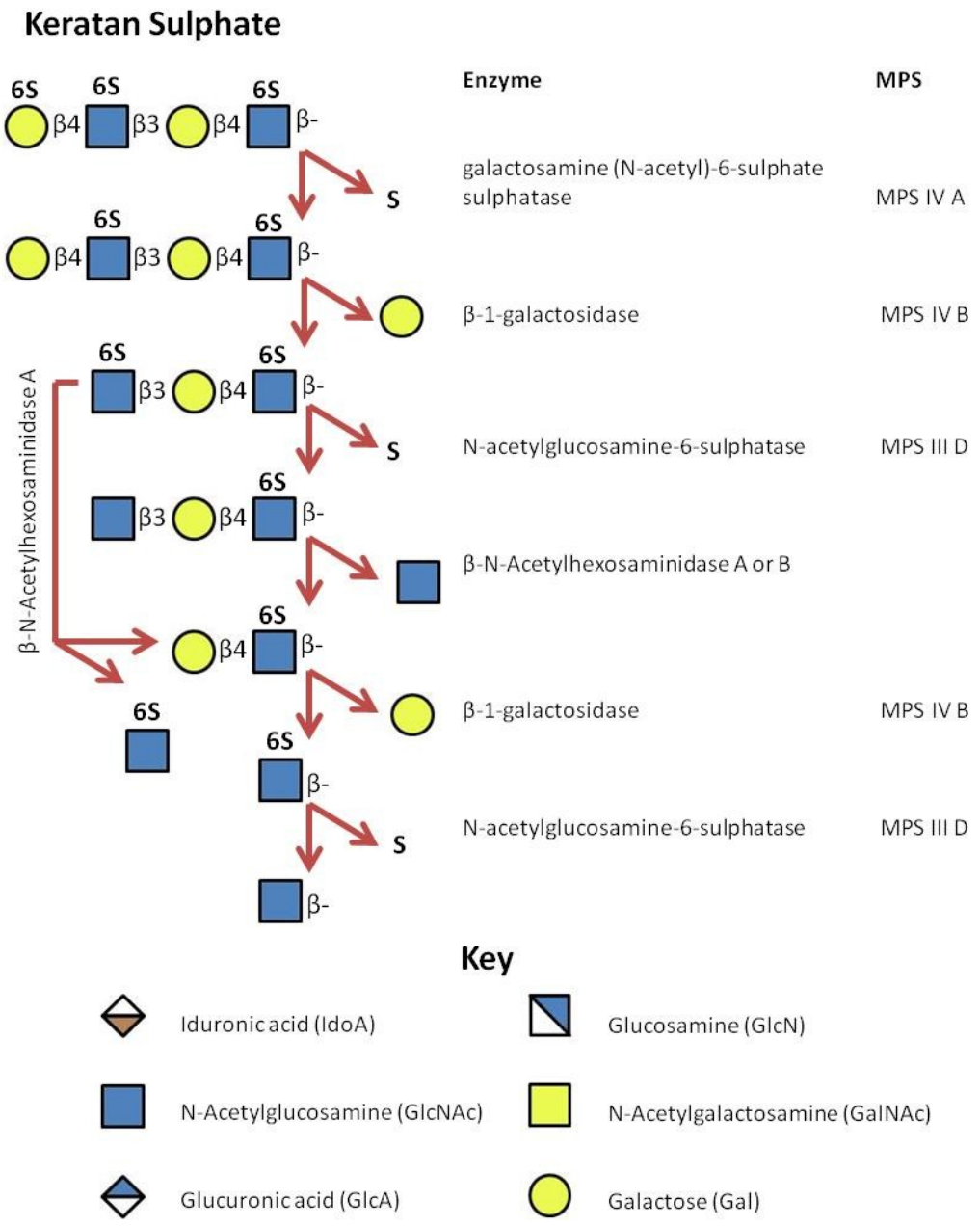


Figure 1-2 The pathway of keratan sulphate degradation

This figure depicts the degradation of keratan sulphate. The enzyme name and MPS disorder resulting from the deficiency of each enzyme are listed next to the relevant reaction. Adapted from Essentials of Glycobiology (Freeze 2009).

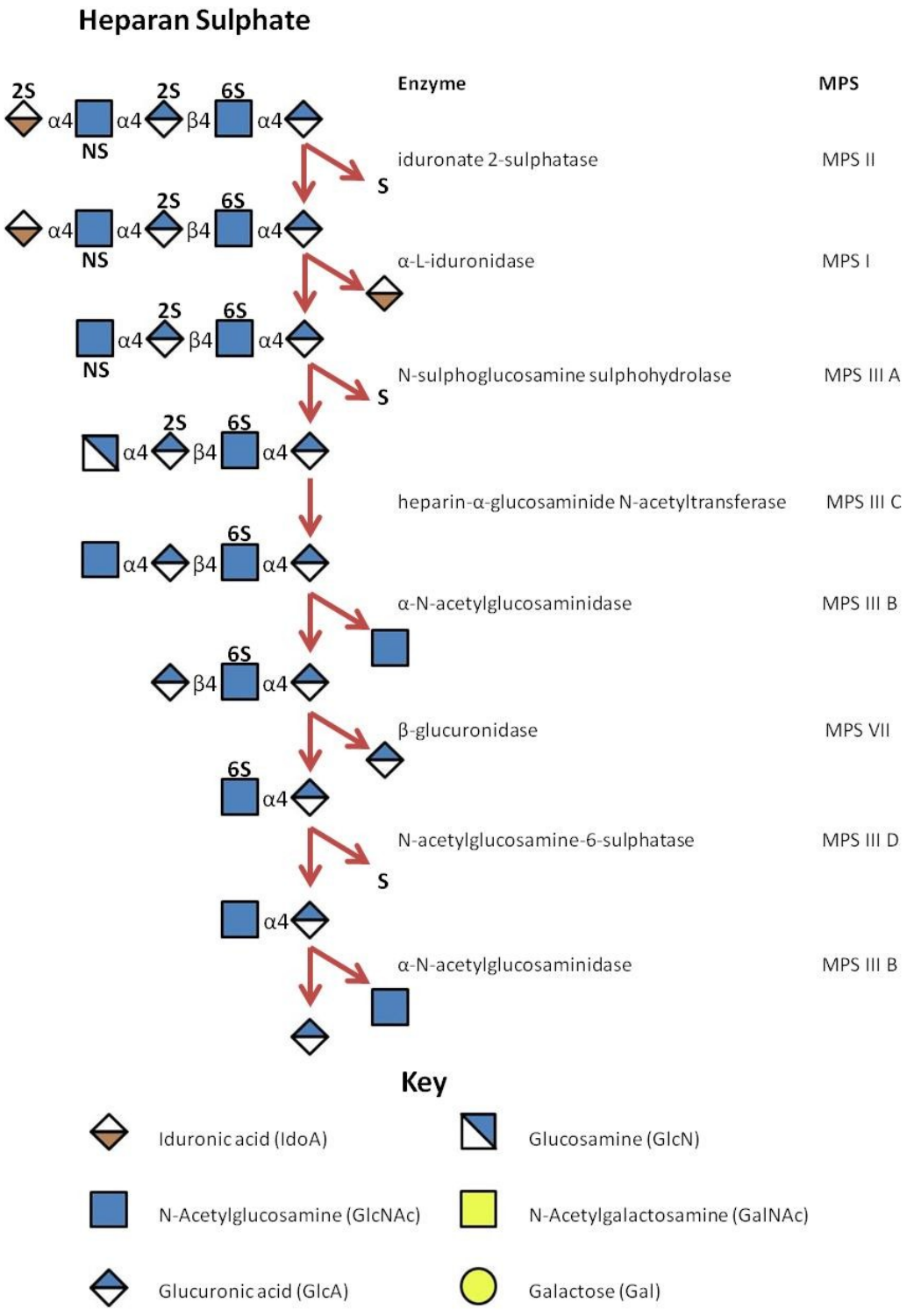


Figure 1-3 The pathway of heparan sulphate degradation

This figure depicts the degradation of heparan sulphate. The enzyme name and MPS disorder resulting from the deficiency of each enzyme are listed next to the relevant reaction. Adapted from Essentials of Glycobiology (Freeze 2009).

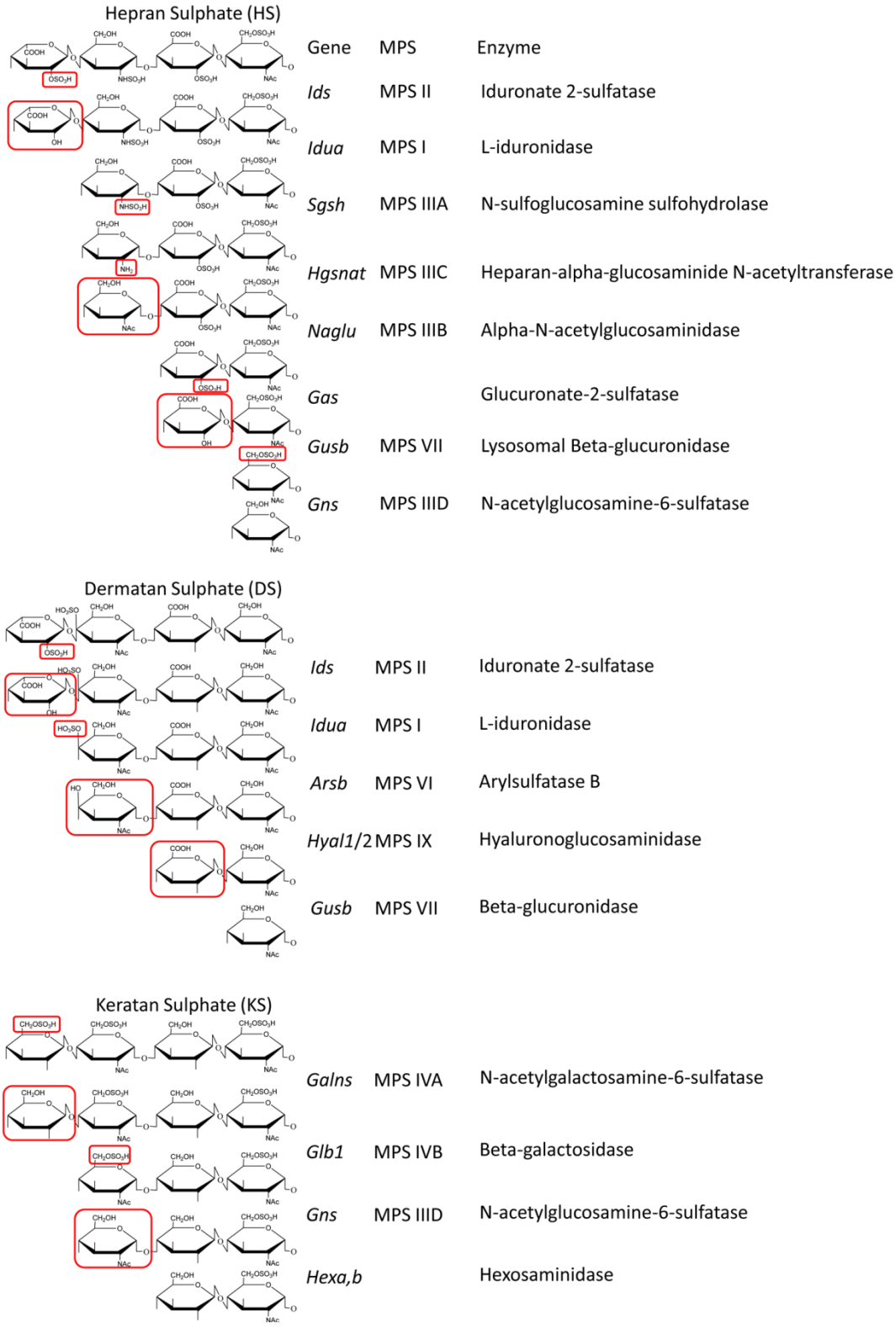


Figure 1-4 Glycominoglycan degradation

This figure depicts the degradation of heparan sulphate, dermatan sulphate and keratan sulphate. The enzyme name and MPS disorder resulting from the deficiency of each enzyme are listed next to the relevant reaction.

1.2.2 Secondary Storage

The secondary storage of GM2 and GM3 gangliosides and un-esterified cholesterol has been observed in MPS III neuronal tissue (McGlynn *et al.* 2004). This secondary storage could result from GAG inhibition of lysosomal enzymes, however the gangliosides often accumulate in different subcellular compartments to the stored GAG. This suggests that altered ganglioside synthesis or trafficking may have an important role in secondary storage (McGlynn *et al.* 2004) possibly caused by Golgi complex disorganisation (Vitry *et al.* 2010). In the brain GM2 and GM3 gangliosides were primarily stored in the piriform cortex, retrosplenial region, amygdala and cortex layer V (McGlynn *et al.* 2004).

Both cholesterol and gangliosides are components of lipid rafts involved in signalling in neurons, leading to the hypothesis that GAG accumulation may affect lipid rafts and therefore signalling in MPS IIIA neurons, causing some of the clinical neurological symptoms (McGlynn *et al.* 2004). GM2 and GM3 gangliosides also have a role in glycosynaptic microdomains that regulate signalling, adhesion, motility and growth which also might be affected (Regina *et al.* 2008).

Recently DS secondary storage has been observed in MPS IIIA, B, C and D patient fibroblasts and potentially occurs in other tissues, although it may not be sufficient to have an effect on the clinical phenotype (Lamanna *et al.* 2011).

1.2.3 Neuroinflammation

Central nervous system (CNS) inflammation has also been linked to neurodegeneration in LSDs (Wada *et al.* 2000; Baudry *et al.* 2003; Jeyakumar *et al.* 2003) including MPS I and IIIB mice (Ohmi *et al.* 2003) and is thought to be an initiating factor for neurodegeneration in Alzheimer's and Parkinson's diseases (Skaper 2007). In MPS IIIA and MPS IIIB mice, activated microglial cells and activated astrocytes have been identified (Savas *et al.* 2004; Malinowska *et al.* 2010)

HS oligosaccharides have been shown to activate microglial cells in MPS IIIB by the TLR4 and MyD88 receptors (Ausseil *et al.* 2008). TLR4 and MyD88 knockout mice were crossed with the MPS IIIB mice; this resulted in a very low level of

neuroinflammation compared to normal MPS IIIB mice at 3 months of age. Despite this, there was no change in progression of neurodegeneration. However neuroinflammation present at 8 months of age was caused by a HS oligosaccharide TLR independent mechanism. This indicates that in MPS IIIB mice neurodegeneration can occur independently of neuroinflammation, at least in the early stages of disease (Ausseil *et al.* 2008).

A recent paper by Arfi *et al*. examined neuroinflammation, oxidative stress and apoptosis gene expression in MPS IIIA mice (Arfi *et al.* 2011). Aspirin treatment reduced the expression of neuroinflammatory genes and oxidative stress genes but had little or no effect on apoptosis related gene expression. This also supports the hypothesis that neuroinflammation may not cause apoptosis and neurodegeneration in MPS IIIA mice. However the pathology of the MPS IIIA mice in terms of primary or secondary storage or behaviour was not examined. Therefore further studies are necessary.

1.2.4 Other Mechanisms

1.2.4.1 Autophagy Defects

Proteins can be degraded by two main pathways, the ubiquitin-proteasome system (UBS) and macroautophagy (autophagy) pathways. The autophagy pathway degrades cytosolic proteins and organelles (e.g. mitochondria) within autophagosomes (double membrane vesicles) which fuse with the lysosome (Mizushima *et al.* 2002). It is hypothesised that in LSDs, neurodegeneration is caused by defective autophagy, since autophagy is a lysosome dependent pathway and mouse model knockouts of autophagy genes result in neurodegeneration (Hara *et al.* 2006; Komatsu *et al.* 2006). Additionally, known autophagy target proteins are accumulated in other neurodegenerative disorders (Settembre *et al.* 2008). The autophagy pathway has been reported to be abnormal in MPS IIIA and Multiple Sulphatase Deficiency (MSD) mouse models as increased numbers of autophagosomes are observed using the LC3II marker (Kabeya *et al.* 2000), more mitochondria and more ubiquitin inclusion bodies are also present (Settembre *et al.* 2008).

There are also parallels with other neurodegenerative diseases. For example, in Alzheimer's disease, lysosomal accumulation is observed (Nixon *et al.* 2008) and autophagy is thought to be an important pathway in the disease (Boland *et al.* 2008). In Huntington's disease and Parkinson's disease the aggregate forming proteins are also targets of the autophagy pathway.

1.2.4.2 Tauopathy

MPS IIIB is also thought to be a tauopathy like Alzheimer's disease; hyperphosphorylated tau was detected in the medial entorhinal cortex and in older mice in the dentate gyrus, but the mechanism of storage is not clear (Ohmi *et al.* 2009). It is not clear if hyperphosphorylated tau in this very limited region of the brain is relevant to the clinical pathology or treatment of MPS. Additionally some of the antibodies against hyperphosphorylated tau used in Alzheimer's disease did not work in Ohmi *et al.* and two prior studies did not observe hyperphosphorylated tau in patients or animal models (Ginsberg *et al.* 1999; Hamano *et al.* 2008).

1.2.4.3 Altered Metabolism

Lysosomal storage has been demonstrated to change the energy usage within the cell and inhibit adipose tissue deposition. The lysosome recycles macromolecules, providing them as raw materials for synthesis; however, if this recycling does not occur then energy will have to be expended for *de novo* synthesis. Additionally, the increasing size and number of lysosomes means that extra energy will be necessary to maintain them. Eventually the increasing demand for energy by the lysosomes and *de novo* synthesis may lead to the cell being unable to carry out its required purpose and eventually it may die (Woloszynek *et al.* 2007).

1.2.5 Behavioural Changes

The primary and secondary storage in MPS III potentially cause neuroinflammation, changes in autophagy and alter metabolism. How these and potently other factors cause the distinct behavioural changes in the MPS III patients is currently unclear. A reduction in VAMP2, a component of the SNAP/SNARE complex that is involved in presynaptic neurotransmitter release and so neuronal signalling, has been observed in MPS IIIB mice (Malinowska *et al.* 2010), which could influence behaviour but many other pathways may also be affected.

When evaluating the behaviour of mice the genetic background can affect the behavioural outcomes. If the colony has a broad genetic background a greater range in behaviours may be seen, which makes determining a significant effect harder. Therefore mice are normally backcrossed on to a standard background such as C57BL/6 (Crawley 2007). The MPS IIIB mouse model was created by targeted disruption of the 6th exon with a 900bp insertion and has also been backcrossed onto the C57BL/6 background (Li *et al.* 1999). The MPS IIIA mouse model is a spontaneously occurring mouse model obtained on a mixed 129SvJ, C57BL/6, SJL, and CD1 background (Bhaumik *et al.* 1999; Bhattacharyya *et al.* 2001). Consequently the MPS IIIA mice should be backcrossed for at least 10 generations onto a pure background for reliable and consistent behavioural testing to be performed (Crawley 2007).

1.2.5.1 Circadian Rhythm

MPS III patients have sleep disturbances; they have trouble settling to sleep and frequently wake up, they also have lower levels of melatonin at night and higher levels during the day (Fraser *et al.* 2002; Fraser *et al.* 2005; Guerrero *et al.* 2006). In the MPS IIIB mice, circadian rhythm changes have also been observed (Heldermon *et al.* 2007; Canal *et al.* 2010). Canal *et al* examined the suprachiasmatic nucleus (SCN), the region of the brain that controls the circadian rhythm, and identified an increase in the size of the lysosomal compartment by LAMP2 immunohistochemistry and an increase in the number of activated microglia and astrocytes. A reduction in vasoactive intestinal peptide (VIP) was observed which suggests that there is a decrease in the transmission of photic information to the SCN from the retinorecipient core. VIP has a role in the resetting of the circadian clock in response to light and is reviewed in Piggins *et al.* and Brown *et al.* (Piggins *et al.* 2003; Brown *et al.* 2007). There were also changes in the distribution of VAMP2 staining from punctate to diffuse that may also indicate a deregulation of signalling within the SCN.

Analysis of the circadian rhythm in the mice is one way to monitor the effect of therapy on disease but it is a lengthy process if a large number of mice need to be examined.

1.2.5.2 Hyperactivity and a Reduced Sense of Danger

Patients present with severe behavioural problems including aggression, hyperactivity and a decreased sense of danger (Cleary *et al.* 1993; Bax *et al.* 1995; Meyer *et al.* 2007; Valstar *et al.* 2008; Heron *et al.* 2011).

MPS IIIB mice are hyperactive in circadian rhythm activity in the home cage in the light (Heldermon *et al.* 2007; Canal *et al.* 2010) or dark (Cressant *et al.* 2004). and in a 10 minute open field test the MPS IIIB mice were also hyperactive (Cressant *et al.* 2004). However in an 8 minute open field test, half in light, half in dark, reductions in activity in both light and dark was observed (Li *et al.* 1999). Another study observed reduced rearing in the second 30 minutes of a 1 hour open field test but did not report overall differences (Fu *et al.* 2007).

Similarly in MPS IIIA mice there are some papers that observe hyperactivity (Hemsley *et al.* 2005; Crawley *et al.* 2006) or hypoactivity (Hemsley *et al.* 2005; Crawley *et al.* 2006; Hemsley *et al.* 2007; Lau *et al.* 2008; Lau *et al.* 2010a; McIntyre *et al.* 2010) in a 3 minute open field test, but often no difference is observed.

Therefore there is a discrepancy in the literature as to whether MPS IIIA and B mice are hyperactive, in line with the patient phenotype, or hypoactive which could correlate with later motor decline in patients.

Mice are a prey species and display thigmotaxis where they avoid open spaces (Simon *et al.* 1994). A reduction in the sense of danger or anxiety can be inferred in mice as a reduction in thigmotaxis behaviour, this can be measured by examining the amount of time or entries into the centre of an open field or on the open arms of the elevated plus maze. A reduced sense of danger in the elevated plus maze test was observed in MPS IIIB mice when performed in the dark (Cressant *et al.* 2004) but not in the light (Fu *et al.* 2007). Therefore it is not clear if the MPS IIIB mice display reduced thigmotaxis or have a reduced sense of danger.

In MPS IIIA mice no difference in the time in centre of the open field was detected by Lau *et al.* (Lau *et al.* 2008), however this could be due to the small size of the open field used. In the elevated plus maze, MPS IIIA mice displayed reduced thigmotaxis or a reduced sense of danger, with a greater proportion of their path

length spent in the open arms at some ages, but no difference at others (Lau *et al.* 2008; Lau *et al.* 2010a). Therefore it is also not clear if MPS IIIA mice display reduced thigmotaxis or have a reduced sense of danger.

Due to the inconsistencies in the field we need to develop a reliable and consistent behavioural test before we can examine the effect of therapies on neurodegeneration in MPS IIIA mice.

1.3 Current Therapies for Mucopolysaccharide and Related Diseases

There are two main strategies for treating lysosomal storage disorders. To increase the rate of breakdown of substrates or to decrease the rate of production of substrate (substrate reduction therapy, SRT) sees Figure 1-5.

The current treatments for MPS are outlined in Table 1-4; they are all treatments that aim to replace the deficient enzyme, either by direct delivery of the enzyme (ERT) or by introduction of a population of cells producing the enzyme. These treatments rely on the process of cross correction.

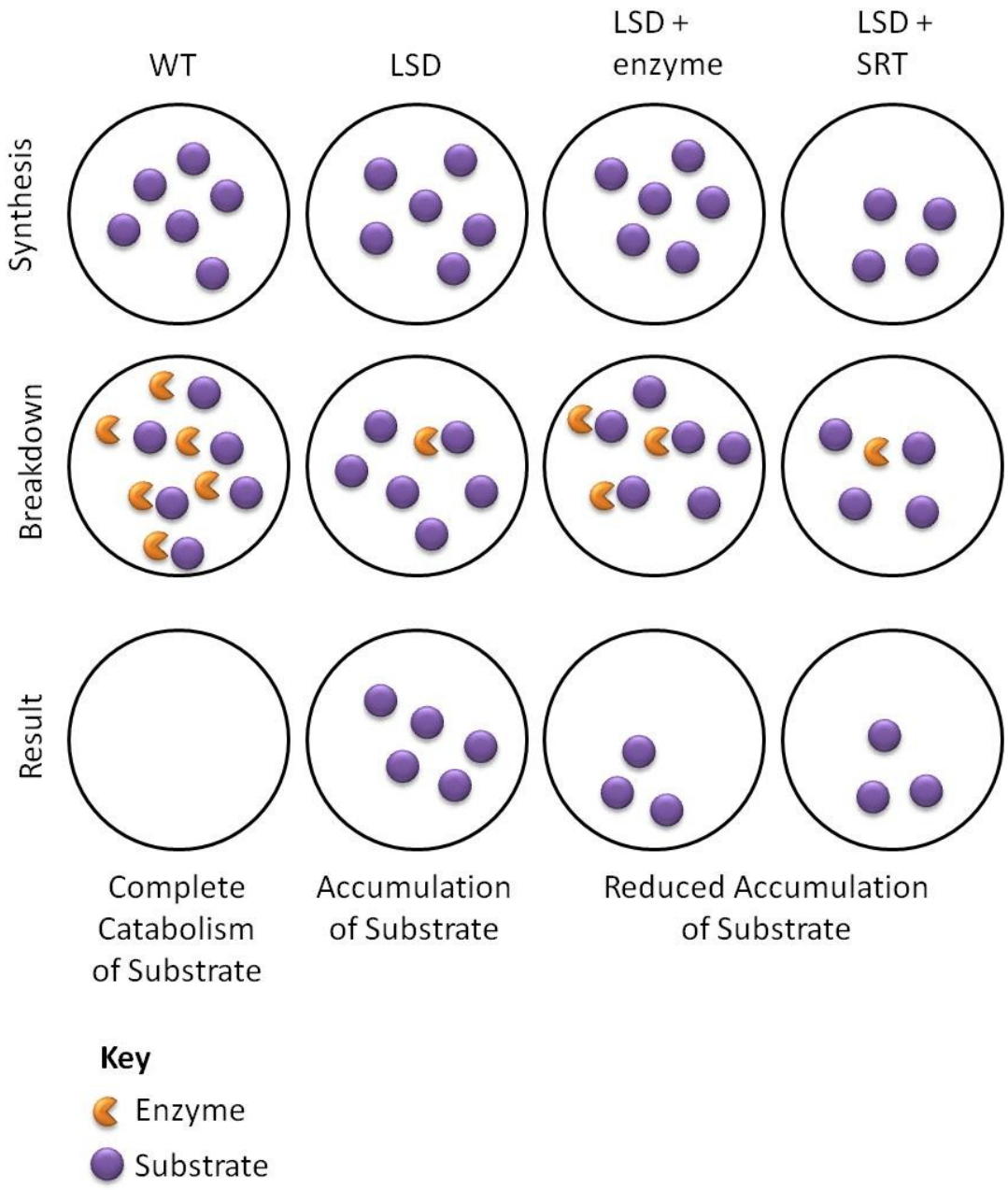


Figure 1-5 Strategies for treatment of lysosomal storage disorders

WT = wild type (normal), LSD = lysosomal storage disorder, SRT = substrate reduction therapy. In normal individuals there is a balance between synthesis and degradation. In lysosomal storage disorders a reduction in the degradation of substrate results in accumulation of substrate. Adding the defective enzyme or factor increases the degradation of the substrate to reduce storage. Alternatively reducing the rate of synthesis to a level that can be degraded also reduces storage of the substrate.

Disease	Alternative name	Standard Treatment
MPS IH	Hurler Syndrome	HSCT, ERT
MPS IH/S	Hurler-Scheie	ERT, HSCT*
MPS IS	Scheie Syndrome	ERT
MPS IIA	Hunter Syndrome	ERT
MPS IIB		
MPS IIIA	Sanfilippo Syndrome A	None
MPS IIIB	Sanfilippo Syndrome B	None
MPS IIIC	Sanfilippo Syndrome C	None
MPS IIID	Sanfilippo Syndrome D	None
MPS IVA	Morquio Syndrome A	ERT
MPS IVB	Morquio Syndrome B	None
MPS VI	Maroteaux-Lamy Syndrome	ERT, HSCT*
MPS VII	Sly Syndrome	None
MPS IX	Hyaluronidase Deficiency	None

Table 1-4 Current standard treatments for MPS
HSCT = Haematopoietic stem cell transplant, ERT = enzyme replacement therapy
*HSCT performed if failed ERT
Adapted from (Prasad *et al.* 2010)

1.3.1 Cross Correction

The mechanism of cross correction is outlined in Figure 1-6. During synthesis of lysosomal enzymes, mannose-6-phosphate (M-6-P) residues are added to sites on the enzyme. The M-6-P then binds to receptors that traffic the enzyme to the lysosome; however, a significant amount of enzyme does not bind to the receptors and is secreted. M-6-P receptors are present on the surface of the cell and take up the enzyme and traffic it to the lysosome, this can happen on the synthesising cell or another cell. This is known as cross correction (Fratantoni *et al.* 1968). Non-receptor mediated internalisation may also occur, although at a much lower level (Sly *et al.* 2006).

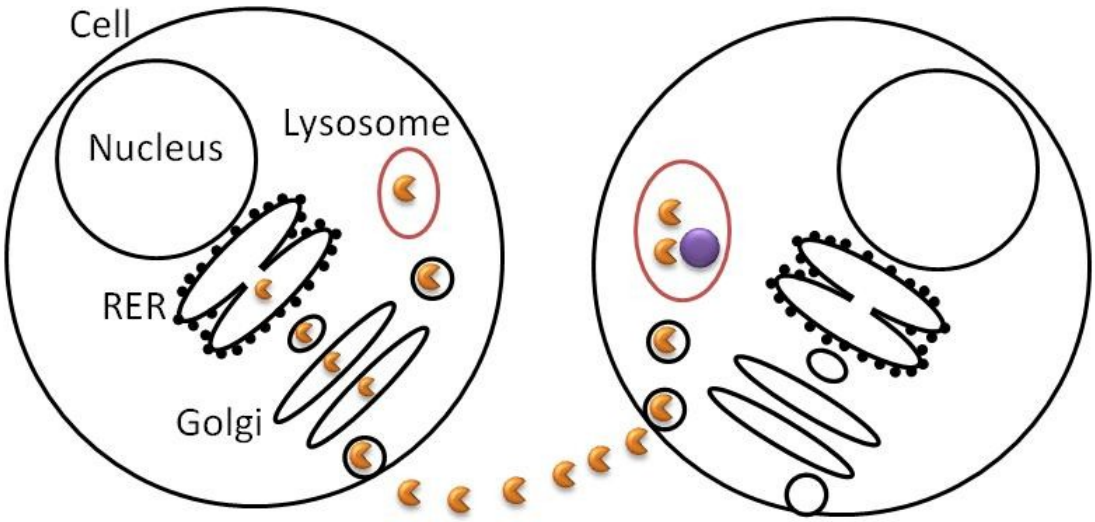


Figure 1-6 Enzyme production and trafficking to the lysosome
Enzyme is produced in the rough endoplasmic reticulum and it is trafficked to the Golgi where mannose-6-phosphate (M-6-P) is added onto the protein. Most of the enzyme is trafficked to the lysosome by M-6-P binding to M-6-P receptors, however some will be secreted. The circulating enzyme can bind to M-6-P receptors on cells which traffic it into the lysosome. Non-receptor mediated cross correction can also occur but at a much slower rate.

1.3.2 Enzyme Replacement Therapy

Enzyme replacement therapy (ERT) is relatively effective at managing non-neuronopathic forms of LSDs e.g. MPS IS. Replacement enzyme is delivered intravenously and taken up by mannose-6-phosphate receptors that are found on the surface of most cells. Barriers to enzyme uptake, such as the blood brain barrier (BBB) and relatively avascular sites, preclude effective distribution of enzyme to sites of clinical need in severe forms of disease, thus limiting the effectiveness of ERT (Figure 1-7). The BBB is made up of endothelial cells bound to each other by tight junctions which actively regulate the passage of macromolecules. Circulating enzymes and many other components of plasma are therefore excluded from the brain. Other regions where cross correction can be problematic include the growth plates of the bone which are poorly vascularised (McIntyre *et al.* 2008).

Non-neuronopathic Gaucher disease was the first LSD to be treated with ERT, and ERT is now available for Fabry and Pompe diseases and MPS types I, II and VI (Germain 2005; Brady 2006; Beck 2007). Systemic ERT is not currently suitable for severe MPS diseases with neurological complications because the administered enzyme cannot cross the BBB. Therefore intravenous ERT is not currently a suitable

treatment for MPS IIIA or any other MPS where insufficient residual enzymatic activity is present in the CNS.

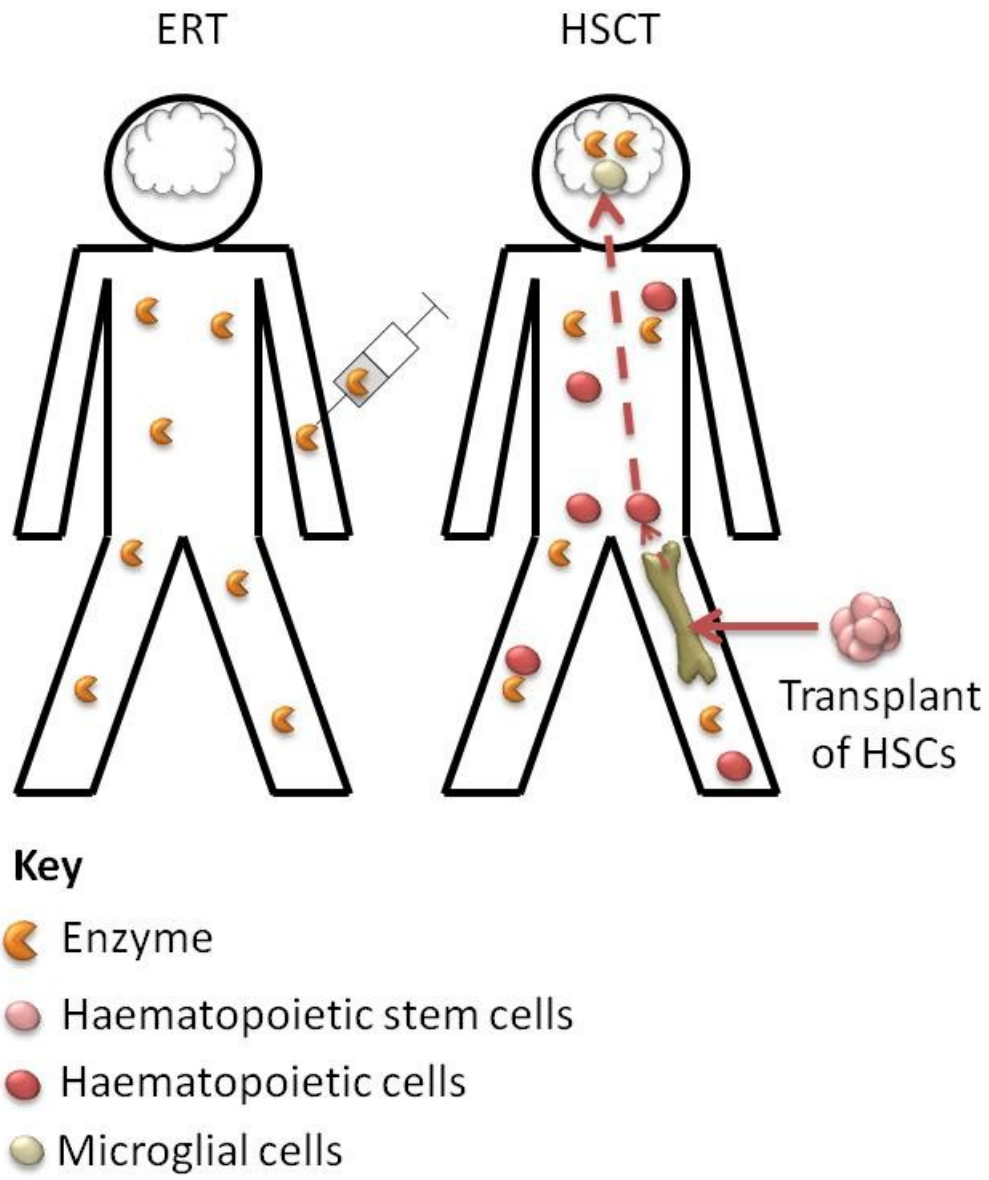


Figure 1-7 Enzyme replacement therapy and haematopoietic stem cell transplant

In enzyme replacement therapy (ERT) recombinant enzyme is delivered intravenously, it is then taken up by other cells by cross correction. However it is unable to pass through the blood brain barrier and little gets to avascular sites like bone growth plates. In a haematopoietic stem cell transplant (HSCT) haematopoietic stem cells (HSC) are transplanted into a recipient that has usually received a conditioning regimen. The HSCs repopulate the haematopoietic system and all these cells produce and secrete enzyme, this can cross correct cells. Circulating enzyme is still unable to cross the BBB but monocytes traffic from the bone marrow to the brain where they differentiate into microglial cells that produce enzyme within the brain that can cross correct neuronal cells.

Weekly intravenous injections of enzyme are prohibitively expensive, in the UK MPS I treatment costs on average £144,000 per patient per year (Wraith 2006). A

humoral immune response can also occur against the administered enzyme, which will reduce the half-life of the enzyme (Wraith *et al.* 2007). Therefore greater doses or more frequent doses will be necessary for effective therapy. In the canine model of MPS I, tolerising animals to exogenous IDUA decreased the production of antibodies and increased efficacy (Dickson *et al.* 2008).

ERT is not suitable to treat MPS IIIA because the neurological phenotype is resistant to ERT due to exclusion of enzyme from the brain by the BBB (Wraith 2006).

1.3.3 Stem Cell Transplantation

Bone marrow transplantation (BMT), also known as haematopoietic stem cell transplantation (HSCT), is designed to provide corrective levels of enzyme and is the only curative therapy available for a small subset of lysosomal storage disorders.

A myeloablative conditioning regimen is necessary to eradicate the patient's bone marrow and marrow derived cells, then bone marrow or cord blood from a suitably matched donor is delivered. The donor cells repopulate the haematopoietic system and produce the deficient enzyme which circulates in the blood and can be taken up by peripheral organs via the M-6-P receptor. Whilst enzyme is unable to cross the BBB, bone marrow derived monocytes traffic across the BBB into the parenchyma of the brain and differentiate into microglia cells. These cells produce enzyme within the brain and mediate neurological cross correction (see Figure 1-7) (Priller *et al.* 2001; Streit *et al.* 2005). Church *et al* showed that in MPS I patients that received HSCT, those patients that received bone marrow from an unrelated donor had a lower urinary DS/CS ratio, which indicates a reduced storage of DS, than those transplanted with heterozygous cells from a related donor (Church *et al.* 2007; Wynn *et al.* 2009b). This suggests that there may be an enzyme dose effect in HSCT in MPS I.

HSCT is currently the most effective therapy for the severe form of MPS I (Hurler syndrome). A recent European study reported that of 93 patients who received cord blood transplants between 1995 and 2007, the three year survival rate is 77%. Of these, 97% achieved full chimerism and of these, normal enzyme activity were achieved in 97% of cases (Boelens *et al.* 2009). A more severe conditioning regimen,

using the chemotherapy drug Busulfan in combination with cyclophosphamide, improved engraftment and survival significantly to 93% after a single transplant (Wynn *et al.* 2009b), and this reflects current clinical outcomes for this disease in the UK.

Despite the similarities between MPS I and III, HSCT is only effective in treating the neurological manifestations of MPS I but not MPS III, where no preservation of cognitive function has been observed (Vellodi *et al.* 1992; Shapiro *et al.* 1995; Sivakumur *et al.* 1999). In MPS IIIA, transplantation with donor umbilical cord blood that is homozygous for normal *SGSH* (Martin *et al.* 2006; Prasad *et al.* 2008; Prasad *et al.* 2010) was found to be more effective than transplantation with donor cells that are heterozygous for *SGSH* (Shapiro *et al.* 1995; Sivakumur *et al.* 1999) but the neurological pathology remains untreated. 12 of 19 children survived the cord blood transplant and the disease was stabilised in 9 patients. The two patients that were transplanted before 2 years of age have exhibited modest gains in cognitive function and their behaviour is slightly improved (Prasad *et al.* 2010). Therefore, it appears that an increase in enzyme activity due to use of a homozygous donor instead of a heterozygous donor can lead to some improvement in disease pathology, but it is probably still insufficient to correct the neurological symptoms. This suggests that there may be an enzyme dose response in MPS IIIA.

In the mouse models of MPS IIIA (Lau *et al.* 2010a) and MPS IIIB (Heldermon *et al.* 2010), bone marrow transplant was unable to correct the abnormal behaviour of the mice. In the MPS IIIB mice only 25% blood donor chimerism was achieved and 1% of normal NAGLU activity in the brain (Heldermon *et al.* 2010). In MPS IIIA mice 90% blood chimerism was achieved. There are several possible reasons for this; donor-derived cells may be unable to home to the brain and secrete enzyme, SGSH and NAGLU may not be secreted or taken up as well as IDUA in MPS I, or the enzyme requirement for a clinically significant response may be significantly higher in MPS IIIA than in MPS I.

1.3.4 Substrate Reduction Therapy

Substrate reduction therapy (SRT) aims to decrease the amount of substrate produced rather than increasing the rate of breakdown of the stored molecule

(Figure 1-5). This could be achieved using small molecule inhibitors that block enzymes involved in the production of GAGs. These inhibitors may be able to cross the BBB and penetrate tissue that does not respond to ERT or BMT. SRT is not currently routinely used in MPS diseases but it is in other lysosomal storage disorders.

The proof of principle for the LSD field is N-butyldeoxynojirimycin (NB-DNJ) (Platt *et al.* 1994), which is an is an inhibitor of glycolipid synthesis, which has been used to successfully reduce storage of GM2 gangliosides in the Tay-Sachs mouse model (Platt *et al.* 1997) and to reduce GM2 ganglioside storage, delay the onset of disease and improve survival of Sandhoff mice (Jeyakumar *et al.* 1999). NB-DNJ (Miglustat) has been approved for mild to moderate non-neuropathic (type I) Gaucher's disease (Cox *et al.* 2000) and more recently Niemann-Pick type C, and while it is less effective than ERT, it is a less invasive and cheaper alternative (Moyses 2003; Zimran *et al.* 2003). It has been tested in a double blind clinical trial (ORPHA87555) in MPS III but was stopped due to a lack of improvement or stabilisation in behavioural and cognitive tests (Vineland adaptive behaviour scale, sleep disturbance questionnaire, hyperactivity measured by Conners scale and the development age determined by the Borel Maisonny Petit test) and despite Miglustat being detected in the brain, no difference was observed in cerebrospinal fluid ganglioside levels (Guffon *et al.* 2011).

Rhodamine B inhibits chain elongation in GAG synthesis by inhibiting sugar precursor formation and/or glycosyl transferase (Kaji *et al.* 1991). This was the first SRT therapy for MPS tested *in vivo* in MPS IIIA mice (Roberts *et al.* 2006). This inhibitor was shown to decrease GAGs in urine, liver and brain, but no reduction in spleen size was observed. However in humans, Rhodamine B has caused mucous membrane and skin irritation and at effective doses it is quite toxic (Dire *et al.* 1987), thus less toxic Rhodamine derivatives will need to be developed and evaluated.

Genistein is an isoflavone that acts as a non-specific protein tyrosine kinase inhibitor. This has been shown to reduce GAG synthesis in MPS I, II, IIIA and IIIB patient fibroblasts by blocking epidermal growth factor receptor (Piotrowska *et al.*

2006). An open-label, pilot clinical study with children suffering from MPS IIIA and B was performed with oral genistein for 12 months. This study revealed no side effects, a significant decrease in urinary GAGs, a change in hair morphology, an improvement in cognitive functions in 80% of patients and stabilisation in 20% of patients. However, there was no placebo control in this study and behavioural measures were based on parents' observations (Jakobkiewicz-Banecka *et al.* 2007). A recent study in our laboratory has demonstrated that in the mouse model of MPS IIIB, long term genistein treatment reduced lysosomal size, heparan sulphate and neuroinflammation in the cerebral cortex and hippocampus and corrected the aberrant behaviour of the MPS IIIB mice (Malinowska *et al.* 2010). A clinical trial is now being planned in Manchester.

Reduced production of heparan sulphate may be an effective therapy for MPS IIIA. However, there is a risk that decreased production of HS will result in production of under-glycosylated proteins, which may cause deficient protein function. Additionally SRT therapy may work best as a supplement to other therapies that increase the degradation of substrate.

1.3.4.1 Substrate Optimisation Therapy

An alternative to substrate reduction is substrate optimisation; in MPS IIIA a disproportionately high proportion of 2-sulphated heparan sulphate is stored. Zacharon Pharmaceuticals have developed a 2-*O* sulphation inhibitor called ZP2345. This inhibitor led to a 30% reduction in HS storage in MPS IIIA mice over a 6 week period. Their hypothesis is that the 2-*O* sulphated heparan sulphate is the hardest form to degrade and by reducing this they have increased the rate of HS degradation (Brown *et al.* 2010).

Therefore substrate optimisation therapy is another small molecule approach that should be able to penetrate the brain and may be beneficial to MPS IIIA patients; however it may work best when combined with a therapy to also increase enzyme activity.

1.4 Therapies in Development

There is currently extensive research investigating different approaches to treat MPS diseases as outlined in Table 1-5, Gene therapy approaches will be discussed in section 1.6 after an introduction to gene therapy.

Therapy	Disease	Pre Clinical	Clinical Trial
Modified ERT – insulin receptor antibody	MPS I	Rhesus monkeys (Boado *et al.* 2008)	
Modified ERT – transferrin	MPS I	Mice (Osborn *et al.* 2008)	
Modified ERT – IGF-II	MPS VII	Mice (LeBowitz *et al.* 2004)	
Modified ERT – amino acid	MPS VII	Mice (Montano *et al.* 2008)	
Intrathecal ERT	MPS I	Rat (Belichenko *et al.* 2005) Dog (Kakkis *et al.* 2004)	NCT00215527, NCT00786968, NCT00852358
	MPS II		NCT00920647
	MPS IIIA	Mouse (Hemsley *et al.* 2008b) Dog (Hemsley *et al.* 2009b)	NCT01299727, NCT01155778
Convection enhanced ERT	Type 2 Gaucher	Rat (Lonser *et al.* 2005)	NCT00244582 (Lonser *et al.* 2007).
	Niemann–Pick A	Mice (Dodge *et al.* 2009)	
Neural Stem Cells	Sandhoff	Mice (Lee *et al.* 2007; Jeyakumar *et al.* 2009)	
Chaperone	Type 1 Gaucher	Fibroblasts (Chang *et al.* 2006)	AT2101 NCT00446550, NCT00813865
	Tay-Sachs and Sandhoff	Fibroblasts (Maegawa *et al.* 2007)	Pyrimethamine, NCT01102686
	Fabry	Mice (Fan *et al.* 2007)	Migalastat NCT00925301
Nonsense mutation read through	Muscular dystrophy	Muscle cells(Welch et al. 2007)	NCT00847379
	MPS I	Cells (Hein *et al.* 2004)	
SRT - Genistein	MPS III	Mice (Malinowska *et al.* 2010)	Low dose (Piotrowska *et al.* 2008) High dose Genistein trial starting 2012 in Manchester
SRT - Rhodamine B	MPS IIIA	Mice (Roberts *et al.* 2007)	
Substrate Optimisation therapy	MPS IIIA	Mice (Brown et al. 2010)	
Anti-inflammatory	Sandhoff and Niemann–Pick type C	Mice (Jeyakumar *et al.* 2004; Smith *et al.* 2009).	
	MPS IIIA	Aspirin, Mice (Arfi et al. 2011)	

Table 1-5 Therapeutic strategies in development for MPS
Examples of current therapies in development for neuropathic MPS and relevant disease where the approach has not been tested in MPS. Partly adapted from (Anson *et al.* 2011)

1.4.1 Receptor Modified Enzyme Replacement Therapy

Enzymes involved in GAG catabolism are unable to cross the BBB due to their large size and non-lipid nature. Various protein fragments that can bind to receptors and enable transfer across the BBB have been coupled to lysosomal enzymes to facilitate their transfer across the BBB. For example, IDUA has been coupled to an anti-insulin receptor antibody that enables active transport across the BBB and into the brain of Rhesus monkeys (Boado *et al.* 2008). Also, transferrin has been coupled to IDUA which facilitated the uptake of IDUA into the brain and, in the short term, reduced stored GAGs in the cerebellum of MPS I mice (Osborn *et al.* 2008).

A fragment of the insulin-like growth factor II (IGF-II) has been fused to β-glucuronidases (GUSB), the deficient enzyme in MPS VII, which allowed enhanced uptake via binding to the bifunctional IGF-II cation-independent M-6-P receptor (LeBowitz *et al.* 2004). Acidic amino acid tags have also been added to GUSB, which enhanced trafficking to bone and brain and increased persistence of the enzyme (Montano *et al.* 2008).

Fc tagged GUSB has been constructed for *in utero* therapy. IgG antibodies are recognised by the Fc receptor, which mediates transcytosis across the syncytial trophoblast layer of the placenta into the foetus. The Fc tagged GUSB was able to pass into the foetus 100 times more than normal GUSB (Grubb *et al.* 2008).

None of these approaches have been attempted for MPS III. However in ongoing work in our laboratory we have observed that 9 out of 10 patients with MPS IH developed high titre antibodies against the enzyme following ERT (Saif, Unpublished data).

1.4.2 Central Nervous System Enzyme Replacement Therapy

ERT delivered directly into the brain via the intrathecal space or cerebrospinal fluid is in development (Savas *et al.* 2004; Hemsley *et al.* 2007; Hemsley *et al.* 2008b; Hemsley *et al.* 2009b). This may be an effective, though invasive therapy for MPS IIIA that targets the CNS. A murine *in vivo* study, funded by Shire Human Genetic Therapies Ltd., demonstrated reduction in storage of GAGs and the number of

ubiquitin-positive lesions. However in the cuneate nucleus region, the number of lesions did not decrease. This may indicate the irreversibility of some changes that occur in MPS IIIA brains (Hemsley *et al.* 2007). The majority of the mice that received the enzyme produced an antibody response against SGSH; this was not associated with adverse effects, although there was some mortality associated with the surgical procedure. A subsequent study used a higher dose of enzyme and demonstrated improved penetration of enzyme into the brain and a decrease in HS storage, the number of lysosomes and activated microglia (Hemsley *et al.* 2008b).

CNS ERT has also been evaluated in a pilot study in the MPS IIIA Huntaway dog model. An increase in SGSH and a decrease in HS was observed in many brain regions however antibodies against recombinant human SGSH were present and moderately severe meningitis occurred (Hemsley *et al.* 2009b). The best results were in one dog that recieved the highest dose (Crawley *et al.* 2011), however this dose was approximately 5 times the dose that will be administered in the high dose of the ongoing clinical trial and approximately 45 times the low dose. Intrathecal ERT has also been evaluated in MPS VI cats with a tolerisation regimen that reduced the production of antibodies against the recombinant enzyme (Auclair *et al.* 2010).

A clinical trial for intrathecal ERT in MPS IIIA has recently started in Manchester, funded by Shire Human Genetic Therapies Ltd (NCT01299727, NCT01155778). Recombinant human SGSH is being administered using an intrathecal drug delivery port either monthly or fortnightly. However the hyperactive behaviour of the MPS IIIA patients is making delivery into the spinal ports difficult. It has also been reported that drug delivery ports can become infected (Boviatsis *et al.* 2004).

1.4.3 Convection Enhanced Central Nervous System Enzyme Replacement Therapy

One of the biggest challenges in CNS ERT is the distribution of enzyme throughout the brain. Convection enhanced delivery is a system to deliver macromolecules in the brain through positive pressure over a longer period of time than a standard injection (Bobo *et al.* 1994). This approach has been evaluated in the Niemann–Pick A mouse model, a decrease in storage of sphingomyelin and a partial improvement in motor function was observed (Dodge *et al.* 2009), and in normal rats and

primates convection enhanced delivery has been used with glucocerebrosidase (the deficient enzyme in Gaucher's disease) where enzyme was widely distributed in the brain (Lonser *et al.* 2005). One neuropathic Gaucher disease patient was treated in a clinical trial of convection enhanced delivery of glucocerebrosidase (NCT00244582), in a 9 month follow up period the patient's neurologic baseline had stabilised and there were no signs of toxicity (Lonser *et al.* 2007). CNS ERT and convection enhanced delivery is reviewed in (Macauley *et al.* 2009).

1.4.4 Neural Stem Cells

In newborn Sandhoff mice, murine and human neuronal stem cells were transplanted. This resulted in an increase in enzyme activity to 4.5% of normal, a decrease in storage and improved survival. These neural stem cells are thought to replace lost neurons and provide a corrective source of enzyme within the brain (Lee *et al.* 2007). In symptomatic Sandhoff mice neural stem cells increased enzymatic activity and improved behaviour, storage of glycosphingolipids and survival (Jeyakumar *et al.* 2009).

None of these approaches have been tested in MPS IIIA, but it is a promising approach; however this approach might work best with autologous neural stem cells that have been transduced by a gene therapy vector to increase expression of the deficient enzyme (see section 1.6.3).

1.4.5 Chaperone

Missense mutations in lysosomal enzymes can result in them being degraded in the endoplasmic reticulum. However chaperones that bind to the enzyme can rescue the enzyme from degradation and facilitate correct folding of the enzyme. Chaperones are typically small molecules so they should be able to cross the BBB and increase enzyme activity within the brain. Chaperones are also typically inhibitors of the enzymes at high concentrations so low concentrations must be used.

The first example of this type of therapy in lysosomal disease is in Fabry disease. 1-Deoxy-galactonojirimycin (DGJ), an active site inhibitor, was administered to Fabry fibroblasts in low concentrations (Fan *et al.* 1999) and to mice expressing human α-

galactosidase containing the R301Q missense mutation, this resulted in an increase in α-galactosidase (Fan *et al.* 2007). DGJ, also known as Migalastat hydrochloride or AT1001, is currently in phase III clinical trial (NCT00925301) (Fan *et al.* 2010). There is also a clinical trial in Type 1 Gaucher using AT2101 (Isofagomine tartrate, NCT00446550, NCT00813865) and in Tay-Sachs and Sandhoff Disease using Pyrimethamine (NCT01102686).

Chaperones could be used to treat those MPS IIIA patients that have missense mutations that benefit from chaperone therapy. 2-acetamido-1,2-dideoxynojirimycin (2AcDNJ) and 6-acetamido-6-deoxycastanospermine (6AcCAS) have been identified as potential chaperones for MPS IIIB (Ficko-Blean *et al.* 2008) and glucosamine is a potential chaperone in MPS IIIC (Feldhammer *et al.* 2009).

1.4.6 Nonsense Mutation Read Through

Nonsense mutations give rise to UAA, UAG or UGA codons in the mRNA, this results in translational termination and mRNA decay (Mendell *et al.* 2001). Initially Gentamycin was used as the pharmacological agent, and results in stop codon read through, however toxic side effects limit the effectiveness of the compound. Gentamycin has been used in MPS I fibroblasts containing Q70X and W402X mutations and successfully increased enzymatic activity (Hein *et al.* 2004).

PTC124 (Ataluren) is a newer compound that induces read through of nonsense mutations and has been evaluated in human muscle cells from muscular dystrophy patients and in mdx mice where it successfully increased dystrophin production (Welch *et al.* 2007). This led to a clinical trial, however the phase IIb study was terminated in 2010 because the primary endpoint of improved walk distance in 6 minutes was not met (NCT00847379). The use of nonsense mutation read through therapy is reviewed in more detail in (Beck 2010; Smid *et al.* 2010).

In summary this approach may be applicable to those patients with suitable mutations but so far it has not been evaluated successfully. However being a small molecule based treatment it is likely to be able to penetrate and treat the brain.

1.4.7 Anti-inflammatory

Neuroinflammation is present in many LSDs including MPS IIIA. In Sandhoff and Niemann-Pick disease type C1 mice, non-steroidal anti-inflammatory (NSAID) treatment delayed the onset of disease and improved the survival (Jeyakumar *et al.* 2004; Smith *et al.* 2009).

In MPS VI rats the anti-TNF-α drug Remicade had a beneficial effect in reducing joint inflammation and pathology (Simonaro *et al.* 2010) and has a greater effect using the rat-specific anti-TNF-alpha drug, CNTO1081, and in combination with ERT (Eliyahu *et al.* 2011). In MPS IIIA mice, 6 months of aspirin treatment reduced the expression of neuroinflammatory genes and oxidative stress genes but had little or no effect on apoptosis related gene expression (Arfi *et al.* 2011). Unfortunately, this study did not examine any other pathological markers of disease or behaviour. Therefore anti-inflammatory treatment in MPS III could be beneficial in combination with a treatment that would reduce the storage of GAGs.

1.4.8 Combination Therapies

The use of non-steroidal anti-inflammatory drugs (NSAIDs) in conjunction with NB-DNJ, a substrate reduction therapy designed to reduce lysosomal storage, has shown a synergistic effect in a mouse model of Sandhoff disease (Jeyakumar *et al.* 2004). The mice lived longer, maintained their coordination and strength longer than untreated controls and had fewer activated astrocytes and microglia in the brain. Similar improvements were also observed in Niemann-Pick disease type C mice (Smith *et al.* 2009)

In the Sandhoff mouse model a synergy was observed with substrate reduction therapy with NB-DNJ or NB-DGJ, and neural stem cell transplant with an improvement in survival (Lee *et al.* 2007). Again, none of these approaches have been tested in MPS IIIA. Additionally HSCT has also been combined with SRT by NB-DNJ in Sandhoff mice and there was an improved life expectancy and motor function, however GM2 gangliosides were not reduced further (Jeyakumar *et al.* 2001).

1.4.9 Gene Therapy

Gene therapy intends to provide a permanent source of enzyme in deficient cells that is sufficient to overcome the enzyme deficiency and may also be able to cross correct other cells (Ponder *et al.* 2007). Gene therapy can be used to incorporate functional copies of the gene into the patient's DNA, which are then transcribed and translated in the long term to produce active enzyme. This approach may provide an extremely powerful treatment for lysosomal storage disorders, and is the focus of this study.

1.5 What Is Gene Therapy?

The main gene therapy approaches include gene replacement, gene repair or gene addition. Gene replacement aims to replace the mutated gene with the correct version of the gene. Low levels of homologous recombination limit the efficacy of this approach (Shesely *et al.* 1991). Gene repair aims to correct the mutated portion of the gene; however, this technology is not currently as advanced as gene addition (Brenner 1999; Parekh-Olmedo *et al.* 2007). The first two approaches have the advantage that the gene is still controlled correctly by the cell.

The third and most popular approach to gene therapy is gene addition. This approach seeks to add a new copy of the gene to the cell where it can be transcribed and translated. The gene can either integrate into the genome or be maintained episomally. Regardless of the approach, gene therapy must achieve several things: i) successful delivery of the gene to the target cells, ii) avoidance of an immune response, iii) localisation to the nucleus, iv) adequate and controlled expression of the target gene(s) and v) low toxicity.

1.5.1 Gene Therapy Vectors

The method of gene delivery to target tissues and cells is an area of intensive research. It can be broadly broken down into two categories, non-viral and viral vector delivery. The differences between the main vectors currently in use are summarised in Table 1-6.

	Retroviral Vector	Lentiviral Vector	Adenoviral Vector	Adeno-associated viral Vector	Liposomes (non- viral)
Genome	RNA	RNA	Linear dsDNA	ssDNA	Circular DNA
Vector Space/kb	6 to 8	6	up to 30*	4.5	20+
Integrates into genome	Yes	Yes	Very rarely	Very rarely	No
Titre (viruses only) (pfu/ml)	$1x10^{10}$	$1x10^{10}$	$5x10^{13}$	$5x10^{13}$	n/a
Expression Duration	Long term	Long term	Transient	Long term	Transient
Infects Non-Dividing cells	No	Yes	Yes	Yes	Yes
Advantages	Stably integrates into the genome	Can transduce non-dividing cells, stably integrates	Large capacity, can transduce non-dividing cells	Can transduce non-dividing cells, less immunogenic than adenovirus	Large capacity
Disadvantages	Can cause oncogenesis, cannot infect non-dividing cells	Smaller than retroviruses, can cause oncogenesis	Transient expression, immunogenic	Small capacity, variable expression	Transient expression, does not integrate, low transduction efficiency

Table 1-6 A comparison of gene therapy vectors
* Gutless vectors

1.5.2 Non-viral Vectors

A non-viral vector needs to be able to successfully deliver the DNA to the target cell without being toxic to the target cell and without the DNA being degraded both before and after it has entered a cell. Therefore DNA is frequently modified or encapsulated to avoid degradation but once inside the cell, the DNA needs to be released to function.

Naked DNA is rapidly degraded *in vivo* by nucleases and is very hydrophilic so it cannot naturally transfect cells. Cationic liposomes, comprised of plasmid DNA surrounded by amphiphilic molecules, contain a positively charged head group which binds to the DNA and a lipid soluble tail that forms the liposome (Felgner *et al.* 1987). They usually have a net positive charge (Behr *et al.* 1989), so that they can bind to negatively charged DNA and to the plasma membrane for internalisation by receptor mediated endocytosis (Escriou *et al.* 1998). Another advantage of the

liposome is that they are resistant to nuclease digestion (Wheeler *et al.* 1999). However, they are not usually targeted to the nucleus, meaning that they must wait for the nuclear envelope to break down on replication before they can be expressed; hence they can only transfect dividing cells. They are not maintained episomally upon cell replication thus genes from these vectors are only expressed transiently.

An alternative to cationic liposomes is polyethylenimine (PEI) which binds to the phosphate of DNA through amino groups. It has a considerable advantage over most liposomal formulations in that it is much more stable in serum as the strong positive charge attracts negatively charged serum proteins. PEI DNA complexes are taken up by receptor mediated endocytosis and PEI pH buffering allows lysosomal escape so that DNA can be transcribed (Boussif *et al.* 1995; Godbey *et al.* 1999). However nuclear targeting is still a problem.

Nuclear targeting has been improved by using DNA with steroid derivatives bound to it. This was then delivered by an adenoviral vector and the steroid derivatives bound to and activated glucocorticoid receptors which then traffic to the nucleus, this is called steroid-mediated gene delivery (SMGD) (Rebuffat *et al.* 2001). This approach has also been used with PEI and the glucocorticoid steroid dexamethasone (Mi Bae *et al.* 2007). The transfection efficacy and nuclear import have been further improved *in vitro* and *in vivo* using triamcinolone acetonide rather than dexamethasone with PEI (Ma *et al.* 2010). However transfection will be transient as the plasmid will not be replicated on cell division.

Persistent expression of non-viral vectors is a challenge, however long term expression has been achieved with the use of scaffold/matrix attachment regions (S/MARs); these sequences are present in the eukaryotic genome and mediate the localisation of genes to the nuclear scaffold. The presence of S/MARs in plasmid DNA has been shown to inhibit chromatin silencing and promote stability and thus providing long term expression (Argyros *et al.* 2008). Expression can also be enhanced by targeting the plasmid to the nucleus, which can be achieved using a nuclear localisation peptide attached to TetR protein that binds to the plasmid TetO

site (Vaysse *et al.* 2004). Targeting the plasmid to the nucleus also confers the ability to achieve expression in non-dividing cells.

The S/MAR approach can be improved by using minicircle vectors. The presence of bacterial genes in plasmids can cause immunogenicity through the presence of prokaryotic CpG islands and potentially silencing of gene expression (Argyros *et al.* 2011). Therefore Cre recombination has been utilised to recombine plasmids into minicircle vectors which lack bacterial proteins, this increases transduction and thus expression (Bigger *et al.* 2001). The use of S/MAR minicircles has enhanced both the persistence and expression *in vivo* (Argyros *et al.* 2011).

An alternative approach to maintain long term expression is to integrate into the genome. Sleeping beauty transposons are a class of non-viral vector that integrate into the genome using SB transposase. These vectors integrate randomly into the genome, although they do favour AT rich regions, and may be able to target specific regions by modifying the transposase with zinc finger proteins (Yant *et al.* 2007).

Zinc finger nucleases are proteins that can direct sequence specific cleavage of DNA. They comprise of DNA sequence specific zinc fingers that bind to the DNA and a cleavage domain derived from *FOK*I. They can be used to perform targeted mutagenesis of a gene by forming a double strand break, or removing a gene by cutting at two points. Alternatively they can be used to repair a gene by homologous recombination. They can also be used to perform targeted gene addition into a specified locus. The use of zinc finger nucleases are well reviewed by Carroll 2011 (Carroll 2011). They are currently in clinical trial to knock out CCR5, the co-receptor for HIV-1, in CD4+ T-cells to produce HIV resistance (NCT00842634 and NCT01044654) (Perez *et al.* 2008). They do however have some drawbacks, including off target DNA cleavage that is potentially toxic and oncogenic. They require the delivery of both the zinc finger nucleases and DNA to the cell, which could be difficult *in vivo* but is less of a problem for *ex-vivo* transduction of stem cells.

1.5.3 Viral Vectors

As a consequence of their natural development, viruses possess many of the characteristics required of a gene therapy vector. They can target cells, insert their DNA, avoid lysosomal degradation and express their genes. Therefore, the modification of viruses to generate gene therapy vectors has been extensively studied and is reviewed in the following sections.

1.5.3.1 Adenoviral Vectors

Adenovirus (Ad) is a double stranded DNA virus, containing a packaging signal and several genes flanked by long terminal repeats. It is a common human pathogen that causes respiratory diseases. Adenoviral vectors only provide transient expression as they are maintained episomally, are not replicated during cell division and have high immunogenicity in humans. In first generation adenoviral vectors the E1 or E3 region was removed, this makes about 3kb available for a transgene. For production E1 must be provided in trans but E3 is not necessary. However expression of the remaining viral proteins elicits an immune response (Yang *et al.* 1996). Therefore in the second generation vector E1 and E3 were removed and E2 and E4 regions were inactivated. Unfortunately *in vivo* immunogenicity persisted due to residual expression of viral proteins. “Gutless” adenoviral vectors were created that contain no viral genes except the long term terminal repeats (LTRs) that allow packaging of the DNA into the virus. In order to produce “gutless” adenovirus, a helper virus is necessary to provide the proteins for virus production. The “gutless” adenovirus maintains gene expression for longer and can accept larger gene inserts (Schiedner *et al.* 1998; Morral *et al.* 1999). Humans have immunity to most adenoviral coat proteins meaning that this response is difficult to avoid. Therefore polyethylene glycol (PEG) has been used to coat adenoviral vectors, which has increased their half life *in vivo* and increased their distribution around the body. The PEGylation decreases the clearance of the vector by antibodies and decreases the activation of the cellular immune response (Eto *et al.* 2008). Adenoviral vector development and utility is reviewed in (Alba *et al.* 2005; Haisma *et al.* 2011).

Adenoviral vectors may be useful in treating cancers where long term expression is not required and first generation adenovirus is easy to grow in high titres. The immune response they elicit can also be harnessed in vaccination. However, they are not ideal for the treatment of genetic disorders such as MPS where long term correction is required. Adenovirus could be re-administered but continual re-administration of the virus will trigger an even more severe immune response.

1.5.3.2 Adeno-associated Viral Vectors (AAV)

Adeno-associated viruses are small single-stranded DNA viruses that belong to the parvovirus family (reviewed in (Buning *et al.* 2008)). They are simple viruses consisting of two open reading frames of rep and cap flanked by inverted terminal repeats. They require the co-transfection with Ad or other viruses for replication. Rep encodes proteins involved in genome replication, transcriptional control, integration and encapsidation of the genome in capsids. Cap encodes three viral capsid proteins VP1, 2 and 3. Modification of these capsid proteins results in different cell tropisms of AAV serotypes. Modifications of this virus to remove the Rep and Cap genes free up around 4kb of space for a transgene and promoter. AAV vector is normally produced by transient transfection of cells with a vector containing the transgene flanked by the ITRs, a plasmid containing rep and cap and an adenoviral helper plasmid. After transfection with the plasmids, the cells are left while vector is produced in the cell and are then lysed to harvest the vector and concentrated by density gradient centrifugation and/or column chromatography.

AAV vectors can transduce non-dividing cells and do not stimulate the immune system to the same extent as adenoviral vectors (Xiao *et al.* 1997). Different serotypes of AAV have different capsids and can be used to transduce different cell types (see Table 1-7) *in vivo*, they are reviewed in (Vandenberghe *et al.* 2009) and (Buning *et al.* 2008).

Initially a specific integration site in the genome on the short arm of chromosome 19 was identified (19q13.3-qter) (Berns *et al.* 1975; Berns *et al.* 1995). Unfortunately, this specificity is present in wild type AAV but is rare in AAV vectors (Rutledge *et al.* 1997) and thus they can cause insertional mutagenesis in neonatal

mice (Donsante *et al.* 2001). However an increase in oncogenesis has not been observed in adult mice (Li *et al.* 2011).

Capsid	Target tissues
AAV1	Muscle, Liver, Joints, Heart
AAV2	Muscle, Liver, Joints, Lung, Eye, CNS
AAV4	Eye, CNS
AAV5	Liver, Lung, Eye, CNS
AAV6	Muscle, Liver, Heart, Lung
AAV7	Muscle, Liver
AAV8	Muscle, Liver, Eye
AAV9	Liver, Heart, Lung, CNS
AAVrh.10	Lung, CNS

Table 1-7 AAV capsid tropism

AAV vector capsid tissue tropism adapted from (Vandenberghe *et al.* 2009)

These vectors have been shown to provide long term expression of factor IX for five months in murine liver (Koeberl *et al.* 1997), expression for 4 years in the canine model of haemophilia by intramuscular injection (Herzog *et al.* 1999) but, in phase II clinical trials using AAV2, a large immune response to this serotype was observed in haemophilia A patients with a commensurately limited correction (Kay *et al.* 2000; Manno *et al.* 2003; Murphy *et al.* 2008). AAV vectors have a limited amount of space for the insertion of therapeutic genes and also have a small risk of insertional mutagenesis. Their expression can be variable and immune responses can occur, although this is dependent on the serotype of the virus. They are currently used in clinical trial in immune privileged sites such as the retina (Buch *et al.* 2008), but with immune suppression during transduction or different capsids they could be more widely applicable. The therapeutic use of AAV is well reviewed in Mingozzi *et al.* (Mingozzi *et al.* 2011).

1.5.3.3 Retroviral Vectors

Retroviral derived vectors are a commonly used gene therapy vector. They can accept an 8 kb insert, integrate randomly into the host genome and are capable of very long term expression. Disadvantages include variable expression, particularly from viral promoters, an inability to infect non-dividing cells and the risk of insertional mutagenesis.

Native retroviruses interact with receptors on the surface of cells via envelope proteins resulting in membrane fusion or phagocytosis. The vector core is released and virus RNA is converted to DNA using the reverse transcriptase enzyme, which the virus encodes. This pre-integration complex then gains access to the host's genome during mitosis when the nuclear envelope breaks down.

Moloney murine leukaemia virus (MoMULV) was the first viral vector that was developed for gene therapy (Dani 1999). The first stage of this process was to remove the viral genes, *gag*, *pol* and *env*, to make space for target gene insertion. The *gag* gene encodes matrix proteins that are necessary for viral replication, *pol* encodes reverse transcriptase, an enzyme that converts the RNA genome of the virus to DNA for insertion in to the host genome, and *env* encodes the envelope protein. *Gag* and *pol* are provided in trans by transient expression from 'helper' vectors, or producer cell lines. *Env* is usually replaced with an envelope from a vector that can infect cells of the same species (ecotropic) or several different species (amphotropic). Removing *gag, pol* and *env* genes also makes the virus replication incompetent as once the retrovirus has infected a cell it will be incapable of producing new viral particles. Sequence homology between the 'helper' vectors and the target gene vector is low to minimise the risk of recombination and thus production of replication competent virus in the host (Markowitz *et al.* 1988b; Markowitz *et al.* 1988a).

1.5.3.4 Lentiviral Vectors

Lentiviruses, including human immunodeficiency virus (HIV), belong to the retrovirus family and have a similar life cycle with some more complex capabilities than retroviruses. Lentiviral fusion occurs in a similar way, but lentiviral pre-integration complexes are imported directly into the nucleus as they hijack nuclear import proteins (Lebeck *et al.* 1992). As before, DNA is integrated into the genome in open chromatin regions where it is transcribed and some of it is translated using the host's cellular machinery to produce proteins needed for new virus formation. Lentiviruses are more complicated than standard retroviruses as they contain additional viral genes encoding regulatory elements transactivator (*tat)* and p19

(*rev)* and genes that enhance pathogenicity including virion infectivity factor (*vif)*, viral promoter r (*vpr)*, viral protein u (*vpu)* and negative replication factor (*nef)*.

As for retroviral vectors, lentiviral vectors have their *gag* and *pol* genes provided in trans. The *env* gene is usually replaced with an envelope from a virus that can infect many different species and cell types – typically the Vesicular Stomatitis Virus G protein (VSV-G). The virulence factors *vif*, *vpr*, *vpu* and *nef* were removed from the genome constructs of the first generation vectors and *gag/pol/rev/tat* and VSV-G *env* were provided in trans on 2 separate plasmids (Figure 1-8). To improve safety in second generation vectors, *tat* can be removed if the 5' viral LTR is replaced with typically a cyclomegalovirus (CMV) promoter. Self-inactivating (SIN) vectors also contain a 400bp deletion in the 3' LTR, which includes the TATA box. This is replicated during viral reverse transcription to the 5' LTR within the transduced cell and results in target gene integration and two deleted LTRs. This improves safety because the viral promoter is disrupted, so new copies of the vector cannot be made. It also reduces the ability for recombination with wild-type HIV and decreases the chance of spontaneously generating a replication competent virus (Zufferey *et al.* 1998).

The vector retains the LTR which contains integration signals and a packaging signal but, most viral genes have been removed to make them replication incompetent and to create space for the gene of interest and an internal promoter to drive expression.

Third generation vectors usually also provide *rev* in trans on a separate plasmid to *gag/pol* which provides a 4 vector production system designed to further minimise the chance of recombination between plasmids.

Other factors have been added to lentiviral vectors to make them more efficient. The woodchuck hepatitis post-transcriptional regulatory element (WPRE) and rev response element (RRE) enhance expression of transgenes by increasing nuclear export by CRM-I (Donello *et al.* 1998; Klein *et al.* 2006). During the synthesis of the second DNA strand after transfection, the central poly-purine tract (cPPT) causes

the production of a 99 nucleotide DNA 'flap' which assists in the nuclear import of the viral genome into the host nucleus (Van Maele *et al.* 2003).

Lentiviral vectors stably integrate into the host genome semi-randomly; they, like retroviral vectors, can cause oncogenesis by activating an oncogene or knocking out a tumour suppressor gene. Lentiviral vectors are less likely to cause insertional oncogenesis because they are more likely to integrate into a gene rather than its promoter region (Schroder *et al.* 2002; Wu *et al.* 2003). Thus are more likely to knock out one of two tumour suppressor genes by integrating into the gene than activating an oncogene by integrating into the promoter.

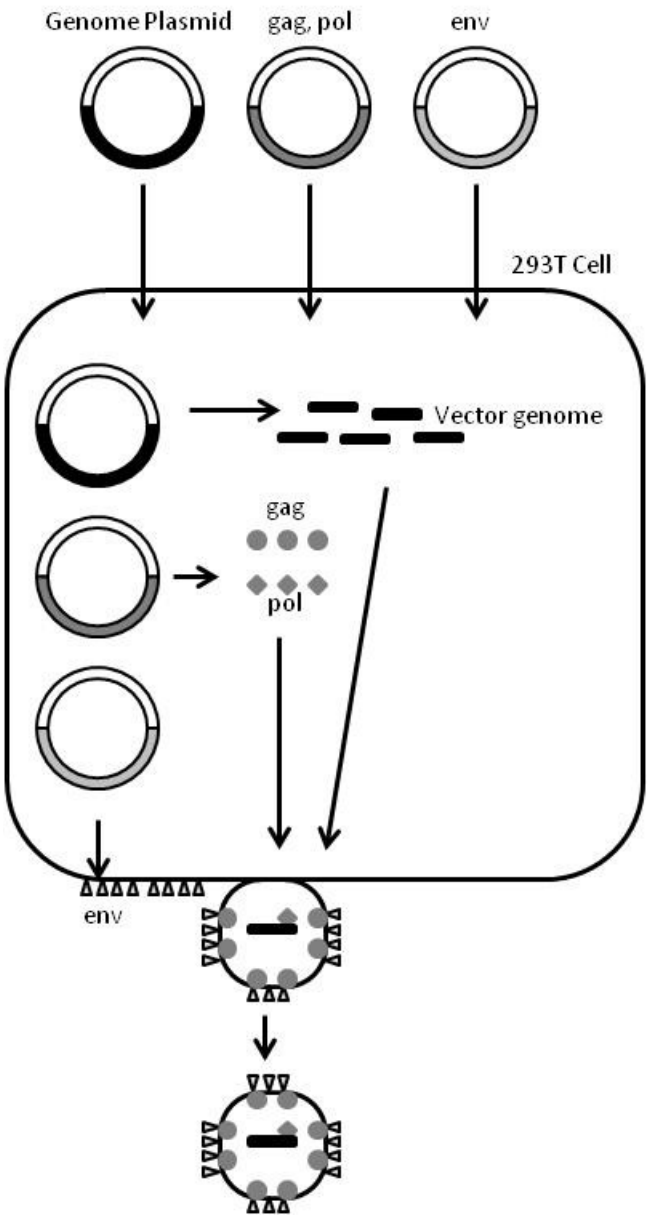


Figure 1-8 Production of lentiviral vector by transient transfection of 293T cells

293T cells are transfected with plasmids, one carrying the lentiviral genome construct expressing the transgene driven by an internal promoter and containing modified LTRs and a packaging signal (Ψ), one containing gag and pol and the other env. The gag and pol genes are expressed from a plasmid under the RSV promoter. The env gene under a RSV promoter (usually VSV-G pseudotype) produces proteins that are incorporated into the plasma membrane. The Ψ signal in the viral genome enables the packaging of RNA into a capsid and the lentiviral vector particle buds off from the transfected cell into the media. The media is collected and centrifuged to concentrate the viral vector particles.

1.5.4 Gene Therapy Safety

The safety of gene therapy is a key concern. However, it is also important to remember the context; if a disease is untreatable, as many genetic disorders are, then the risks associated with the therapy become acceptable. Several possible risks are associated with gene therapy, some of which apply solely to the use of

viral vectors. There may be a large immune response to the vector, as occurred in an adenoviral gene therapy trial for ornithine transcarbamylase deficiency (Marshall 1999; Wilson 2009). The vector could be or become replication competent, allowing it to infect many cells in the patient with potentially toxic effects, although this is unlikely as all vectors are tested for the inability to replicate prior to testing in patients. Integrating vectors could insert into the genome disrupting a tumour suppressor or promoting an oncogene and cause cancer and germline transduction.

Retroviral vectors have been utilised in the current clinical successes of X-SCID (X-linked severe combined immunodeficiency) gene therapy (Hacein-Bey-Abina *et al.* 2003). X-SCID is an inherited genetic disorder caused by mutations in the cytokine receptor common γ chain (IL2RG) resulting in very low T-cell numbers. Patients can be treated with a bone marrow transplant however suitable donors do not always exist. There have been two X-SCID clinical trials, one based in France lead by Marina Cavazzana-Calvo and Alain Fischer and the other in the UK lead by Adrian Thrasher and Bobby Gaspar. In both cases autologous CD34+ HSCs were modified to express IL2RG using a retroviral vector. Nineteen out of the twenty patients across the two trials were treated successfully (Cavazzana-Calvo *et al.* 2007; Gaspar *et al.* 2011a). However, the insertion of the gene into the host genome caused activation of oncogenes and resulted in leukaemia in five of the patients, of which one has died but the other four have recovered their T-cell repertoire and are essentially leading normal lives (Hacein-Bey-Abina *et al.* 2003; Fischer *et al.* 2011). Bone marrow stem cells are mostly non-cycling (Lord *et al.* 1993) and are therefore more difficult to transduce using retroviruses (Miller *et al.* 1990) therefore the HSCs in this trial were transduced three times (Cavazzana-Calvo *et al.* 2000; Gaspar *et al.* 2004) to allow for sufficient transduction. This may have led to selection of the fastest growing cells and cells with more leukaemic potential. Additionally, the oncogenesis was associated with integration into LIM domain only 2 (LMO2) locus and appeared to be specific to X-SCID, as in ADA-SCID trials there was not the preference for LMO2 integration and no oncogenesis has occurred (Ferrua *et al.* 2010; Fischer *et al.* 2011). Leukaemia has also occurred in one patient who recieved *ex vivo* retroviral gene therapy for Wiskott-Aldrich syndrome (WAS), which was also associated with

the LMO2 locus (Fischer *et al.* 2011). Therefore self inactivating (SIN) vectors have been designed that contain an internal promoter rather than using one in the LTR to drive expression of the transgene. This reduces the activation of genes neighbouring the integration site (Yu *et al.* 1986).

Additionally it has become clear that retroviral integration is not a completely random process. Using the retrovirus murine leukaemia virus (MLV) and the human cancer HeLa cell line, it appears that retroviruses preferentially insert into transcriptional start regions. This can lead to activation of oncogenes and disruption of tumour suppressor genes (Wu *et al.* 2003). A lentiviral vector was used to transfect the human lymphoid cell line SupT1. Insertional analysis revealed that lentiviruses appear to insert preferentially into active genes and local hotspots. Therefore, lentiviruses may have a lower risk of insertional oncogenesis than retroviruses, as they integrate within a gene they are less likely to activate an oncogene. Lentiviral integration may still disrupt tumour suppressor genes but it is preferential to disrupt one copy of two of a tumour suppressor gene rather than activating an oncogene (Schroder *et al.* 2002).

A study of 3127 integration sites from HIV, MLV and avian sarcoma-leukosis virus (ASLV) in human cells, confirmed that lentiviruses have a preference for active genes, and retroviruses tend to integrate into the promoter regions of active genes (Mitchell *et al.* 2004). The MLV retrovirus favoured integrating into the CpG islands (regions where a cytosine nucleotide is followed by a guanine nucleotide) which are found in 72% of human promoters (Saxonov *et al.* 2006) but are sparsely located elsewhere in the genome. Lentiviruses appear to avoid these CpG islands (Mitchell *et al.* 2004).

The risk of causing an immune response that causes harm to the patient or decreases the efficacy of the therapy is an important consideration. To reduce this risk it is advisable to choose use a less immunogenic vector that reduces the expression of viral proteins.

1.6 Gene Therapy for MPS Disease

MPS, like other lysosomal disorders, is a good target for gene therapy because tight regulation of the gene is not likely to be needed and cross correction occurs, therefore all cells do not need to be transduced for enzymatic correction to take place. There are several different delivery routes outlined in Figure 1-9 and Table 1-8.

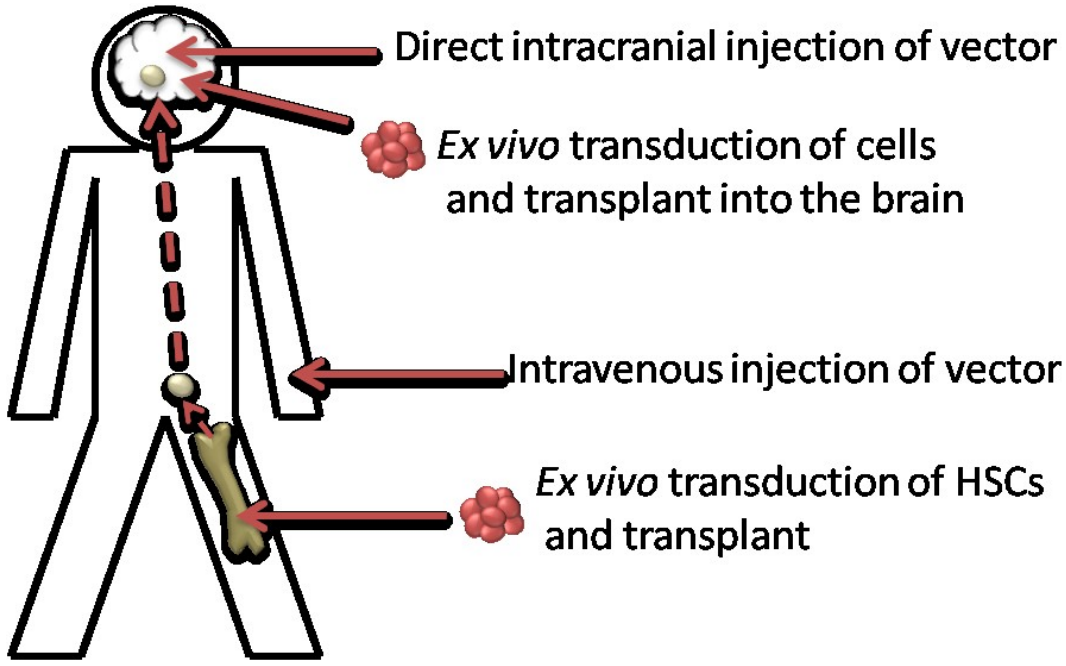


Figure 1-9 Routes of gene therapy delivery

Therapy	Disease	Pre Clinical	Clinical Trial
Intravenous- non viral	MPS I	(Aronovich *et al.* 2007; Aronovich *et al.* 2009; Osborn *et al.* 2011)	
	MPS VII	(Aronovich *et al.* 2007; Richard *et al.* 2009)	
Intravenous- viral	MPS I	(Hartung *et al.* 2004; Di Domenico *et al.* 2005; Kobayashi *et al.* 2005; Liu *et al.* 2005b)	
	MPS IIIA	(Anson *et al.* 2007; McIntyre *et al.* 2008; McIntyre *et al.* 2010)	
	MPS VII	(Daly *et al.* 2001; Stein *et al.* 2001)	
Intravenous- BBB Permeabilising - Mannitol	MPS IIIA	(Anson *et al.* 2007; McIntyre *et al.* 2008)	
	MPS IIIB	(McCarty *et al.* 2009)	
Intravenous –BBB Penetrating Vector	MPS IIIB	(Fu *et al.* 2011)	
Intravenous –BBB transduction	MPS VII	(Chen *et al.* 2009)	
Gene therapy – CNS	MPS I	AAV Mice, (Desmaris *et al.* 2004), Dog (Ciron *et al.* 2006), Primate (Ciron *et al.* 2009)	
	MPS IIIA	Lentivirus (McIntyre *et al.* 2010), AAV (Fraldi *et al.* 2007)	AAV SAF-301 NCT01474343 Started 2011
	MPS IIIB	AAV (Fu *et al.* 2002; Cressant *et al.* 2004; Fu *et al.* 2007; Fu *et al.* 2010) Lentivirus (Di Domenico *et al.* 2009)	AAV estimated starting 2012
	MPS VII	AAV (Frisella *et al.* 2001; Liu *et al.* 2005a; Cearley *et al.* 2007) Lenti (Brooks *et al.* 2002; Bielicki *et al.* 2010)	
	Battens Disease	AAV (Passini *et al.* 2006)	NCT00151216 (Worgall *et al.* 2008), NCT01161576
Gene therapy - HSCT	MPS I	Retrovirus (Zheng *et al.* 2003)	
	MPS I	Lentivirus (Visigalli *et al.* 2010)	In planning stage
	MPS IIIA	Lentivirus – this thesis	
	MPS IIIB	Retrovirus (Zheng *et al.* 2004)	
Gene therapy - microglia	MPS IIIA	(Robinson *et al.* 2010)	
Gene therapy - neural stem cells	MPS VII	(Meng *et al.* 2003)	
Gene therapy – stromal cells	MPS VII	(Sakurai *et al.* 2004)	
Gene therapy – macrophages	MPS VII	(Ohashi *et al.* 2000)	

Table 1-8 Gene therapy strategies in development for MPS
Examples of gene therapies in development for neuropathic MPS and relevant diseases where the approach has not been tested in MPS. Partly adapted from (Anson *et al.* 2011)

1.6.1 Intravenous Gene Delivery

The easiest route of delivery of gene therapy vector is by intravenous injection.

1.6.1.1 Intravenous Non-viral Gene Therapy

In non-viral gene therapy, plasmids need to be assisted in entering cells unlike viral vectors. One method is to deliver plasmid in a large volume very rapidly via the tail vein in rodents (Budker *et al.* 1996; Hodges *et al.* 2003). This method is called hydrodynamic delivery and results in transfection of the hepatic cells with the plasmid. Plasmid DNA containing the GUSB gene was delivered hydrodynamically via the tail vein of MPS VII mice in a volume equivalent to 10% of the mouse's weight, over 5 seconds (Richard *et al.* 2009).

This resulted in a high level of GUSB expression in the liver and 100-500% of normal activity in the serum at day 2-8, but by 60 days post injection less than 5% of normal activity remained. There was a significant reduction in GAG storage in the liver, spleen, kidney, and critically, in the brain 28 days after injection. Despite the significant increase in GUSB enzyme activity to 1% in the brain and the decrease in GAG storage by 50%, there was poor correction of cellular storage suggesting that the enzyme activity in the brain were insufficient. It was demonstrated that the GUSB activity found in the brain was not due to the presence of plasmid in the brain as there was no increase in GUSB mRNA levels in the brain, therefore the enzyme must have crossed the BBB. This uptake could have been due to the high levels of circulating enzyme saturating the few M-6-P receptors that are present or by a phagocytic process (Richard *et al.* 2009).

To improve the persistence of gene expression, minicircles can be used to reduce gene silencing and reduce the immune response by removing pro-inflammatory hypo-methylated CpG motifs found in bacterial DNA. This has been evaluated in MPS I mice to provide long term expression (Osborn *et al.* 2011). The minicircle DNA was delivered by hydrodynamic tail vein injection and CD4 and CD8 depleting antibodies were used for immune suppression. The storage of GAGs in the visceral organs was reduced to normal levels and enzyme was expressed for 100 days with a 10 fold drop in expression, however, further evaluation of this therapy is necessary to assess the therapeutic effect on the brain.

An alternative method to increase the duration of gene expression is to use sleeping beauty transposons. After hydrodynamic delivery into MPS I and VII mice the DNA integrated into the genome using the sleeping beauty transposon. After 3 months high expression (1 to 100 fold of WT levels) was observed (Aronovich *et al.* 2007), however cyclophosphamide immune suppression was required. This approach was evaluated in NOD/SCID MPS I mice, where high expression was also achieved and detectable IDUA activity in the brain was associated with a reduction in GAG storage (Aronovich *et al.* 2009). It was thought that some enzyme was able to pass through the BBB due to the high levels of circulating enzyme.

The hydrodynamic plasmid delivery system used to transduce the liver in mice has recently been shown to be applicable in transfecting skeletal muscle and the liver in pigs (Kamimura *et al.* 2009; Kamimura *et al.* 2010). Therefore this seems like a safe approach to transfect these organs and potentially other organs, however it is unlikely to be applicable to the brain as transfection of the liver results in a transient 2.5 fold increase in size that would be damaging to brain tissue.

1.6.1.2 Intravenous Viral Vector Gene Therapy

Intravenous gene therapy with retroviral or lentiviral vectors in adult MPS I mice has been unsuccessful. Unstable expression and low enzymatic activity have been observed (Di Domenico *et al.* 2005; Liu *et al.* 2005b). In MPS VII mice, a feline immunodeficiency viral vector was administered intravenously and a reduction in the level of GAGs was found in the liver, where most cells were transduced, but the overall clinical effect was limited (Stein *et al.* 2001). A recent MPS IIIA intravenous lentiviral gene therapy study has been more successful, showing 186% and 68% of normal SGSH activity in spleen and liver respectively. Immunohistochemistry with toluidine blue showed normalisation of GAGs in the liver, spleen and heart, but not chondrocytes. Levels of hexosamine-N-sulphate [α-1,4] hexuronic acid (HNS-UA), a marker of HS storage, in the brain were also decreased but no SGSH activity was detected there. Additionally, no behavioural observations were made, nor the effect on neurons recorded (Anson *et al.* 2007; McIntyre *et al.* 2008). In a further study by McIntyre *et al,* intravenous gene therapy had no effect on the storage of HS in the brain (McIntyre *et al.* 2010).

The immaturity of the newborn immune system may allow stable expression of gene therapy vectors in mice, and neonatal mice still express M-6-P receptors on the BBB allowing enzyme passage into the brain (Urayama *et al.* 2008). Neonatal gene therapy by AAV, lentiviral and retroviral vectors has produced long-term vector expression and an improvement in disease phenotype in MPS I (Hartung *et al.* 2004; Kobayashi *et al.* 2005; Liu *et al.* 2005b) and MPS VII mice (Daly *et al.* 2001). However, neonatal gene therapy is not always an option, so alternative delivery methods should be considered.

Intravenous administration of the gene vector may produce sufficient circulating enzyme activity to treat the peripheral tissue phenotype. Whether significant passage of enzyme across the BBB in MPS III can occur when there is a large excess of enzyme in the blood is unknown, but there is some evidence to suggest that it can cross the BBB in MPS I and VII mice (Urayama *et al.* 2004; Vogler *et al.* 2005; Urayama *et al.* 2008; Aronovich *et al.* 2009). Additionally the vector or enzyme may elicit an immune response that limits their expression.

1.6.1.3 Intravenous Viral Vector Gene Therapy – Permeabilising the BBB

To enhance enzyme expression in the brain it would be advantageous to transduce some of the cells within the brain to provide a source of enzyme that does not have to pass through the BBB. One way to do this is to use mannitol, an osmotic BBB disruption agent, that is used in patients and has been used to facilitate AAV uptake into the brain in rats (Mastakov *et al.* 2001). It has also been used in the MPS IIIB mouse model. Delivery of AAV2, 8 minutes after mannitol allowed transduction of cells in the brain. The timing of the dose was critical and 3,000 fewer cells were transduced if 20 minutes elapsed between mannitol and AAV injection. This was associated with a reduction in HS storage in the brain and behavioural correction (McCarty *et al.* 2009). It has also been shown to enhance transduction and distribution of intracranial injected AAV (Fu *et al.* 2007). However the use of mannitol with intravenous lentiviral vector has not been shown to have a clear benefit in MPS IIIA mice (Anson *et al.* 2007; McIntyre *et al.* 2008).

This approach may be a minimally invasive approach to transduce some of the cells in the brain and correct the periphery. However an immune response may be raised

against the AAV capsid or the enzyme which may limit duration of expression and cause side effects.

1.6.1.4 Intravenous Viral Vector Gene Therapy – Penetrating the BBB

Another approach is to use AAV serotype 9. After intravenous delivery this AAV vector is able to pass through the BBB and transduce neuronal cells in neonates and astrocytes in adult mice (Zincarelli *et al.* 2008; Foust *et al.* 2009). This has also been demonstrated in cats where motor neurons were also reported to be transduced (Duque *et al.* 2009). This vector has been evaluated in the mouse model of MPS IIIB and also in combination with mannitol (Fu *et al.* 2011). In the brain, neuronal, glial and endothelial cells were transduced and GAG storage was reduced. This was associated with increased lifespan and improvements in motor behaviour. Mannitol pre-treatment also improved transduction in the brain.

This promising approach has not been assessed in MPS IIIA. As with other intravenous approaches the disadvantage is the possibility of an immune response against the vector or enzyme. It will also need to be demonstrated that this vector will have the same tropism in patients.

1.6.1.5 Intravenous Viral Vector Gene Therapy – Transducing the BBB

Rather than trying to penetrate the BBB, another approach is to transduce the endothelial cells of the BBB (Chen *et al.* 2009). The brain is a highly vascularised organ and if you could selectively transduce the brain endothelia, these cells could secrete enzyme into the blood and into the brain to cross correct neuronal cells. A phage display library was injected intravenously into WT and MPS VII mice, the brain was then removed and the phage purified and re-injected five times, an approach known as phage panning. The DNA was sequenced from the phage and the common peptide sequences identified. Peptide sequences that bound to the MPS VII brain but not the WT brain were selected and cloned into the AAV capsid. This AAV was then administered to MPS VII mice with β-glucuronidase. Transduction of the brain endothelium was 35 fold higher than liver and better than unmodified AAV. This reduced the number of cells with storage vacuolation and β-glucuronidase staining co-localised with neuronal staining suggesting that neuronal cells had been cross corrected.

This approach has not been assessed in MPS IIIA and it has not yet been determined if these peptide sequences will enhance brain endothelium transduction in patients or if specific targeting peptides will be needed for each disease and different ones between mouse and human.

Intravenous delivery is the easiest route of gene therapy vector delivery, however this results in production of enzyme in the body not the brain and it is not clear if sufficient enzyme can cross the BBB even at high peripheral circulating levels. Therefore for some approaches, mannitol or selective transduction of the brain endothelium could be applicable or a viral vector with trans BBB ability could be used. However intravenous delivery rapidly brings the vector into direct contact with the immune system, which could limit transduction or cause a dangerous immune response. An immune response against transduced liver cells would not be too bad as it has a high repair capacity but this is not true of all tissue, especially neurons.

1.6.2 Intracranial Gene Therapy

In order to overcome the problem of the BBB inhibiting the passage of enzyme into the brain from the body, direct gene therapy into the brain has been researched. Adeno-associated virus has been used in the MPS IIIA mouse model to directly express SGSH in the CNS (Fraldi *et al.* 2007). Only those regions of the brain that were effectively transduced, including the striatum, cerebral cortex and olfactory bulb, showed a reduction in storage. When comparing brain slices as a whole for a disaccharide marker of HS storage, HNS-UA (King *et al.* 2006), no significant reduction was observed. More recently the use of a lentiviral vector expressing SGSH has been evaluated and a greater increase in SGSH activity and decrease in storage has been observed (McIntyre *et al.* 2010). Treating newborn MPS IIIA mice with a canine adenoviral vector expressing SGSH was more effective and a significant decrease in vacuolation and an increase in SGSH activity was observed but expression duration was limited (Lau *et al.* 2010b). The difference in size between murine and human brains and the limited penetration of the virus into the brain may be the major limiting factors for this therapy, and could also account for the better response of newborn mice which have smaller brains. The serotype used and the location of injections is important, it has been demonstrated in MPS VII

mice that AAV9 was capable of transducing neuronal cells and that the vector spread down axonal pathways out of the region the injection. This increases the area of the brain that could be corrected from each injection site (Cearley *et al.* 2006).

A murine MPS IIIB study using an adeno-associated viral vector to express NAGLU and directly injecting into the striatum, has successfully decreased GM2 and GM3 ganglioside storage and improved the behaviour of the mice (Cressant *et al.* 2004; Heldermon *et al.* 2010). This approach has also been evaluated in MPS I and VII mice (Frisella *et al.* 2001; Desmaris *et al.* 2004; Cearley *et al.* 2007).

Intracranial gene therapy using AAV has also been evaluated in the Sandhoff disease mouse model. Two injections per hemisphere into the striatum and cerebellum at 4 weeks of age resulted in an improvement in survival from under 20 weeks to over a year. This was associated with delayed disease onset, preservation of motor function and a reduction in GM2 ganglioside storage (Cachon-Gonzalez *et al.* 2006).

There is a current clinical trial of intracranial gene therapy (NCT01474343) in MPS IIIA using AAV10 containing *SGSH* and *SUMF1* and a similar trial is planned for MPS IIIB to start in 2012. This therapeutic approach has potential but may need to be combined with another gene therapy protocol, ERT or SRT, to provide enzyme for peripheral tissues or reduce substrate production to treat the mild somatic manifestations of the disease. Also, this therapy carries the risk of neurological damage and the effects of gene therapy in the brain are currently unknown.

There has been a clinical trial (NCT00151216) in late infantile neuronal ceroid lipofuscinosis (a form of Battens disease) that used 1.8-3.2 x 10^{12} AAV2 vector particles per patient in 12 locations in the brain from 6 burr holes (3 per hemisphere) (Worgall *et al.* 2008). A low level of anti AAV2 capsid antibodies were detected in 4 of 10 patients. Of the 10 patients one developed status epilepticus and died 49 days post surgery, it could not be determined if this was related to the surgery, vector or the underlying disease. However there was no evidence of CNS inflammation. There were 60 serious adverse events of which 34 were possibly

related to the vector or surgery but it was hard to determine for certain. However the trial was successful in reducing the rate of decline compared to controls in the neurologic rating scale. A new clinical trial is now underway (NCT01161576) using the rh.10 Rhesus Macaque-derived Adeno-associated Virus Vector (AAVrh.10) rather than AAV2 as higher expression and a better distribution of AAV vector has been observed in rats and mice using this vector (Sondhi *et al.* 2007).

Aside from the safety of this therapy, the biggest challenge is getting correction throughout the brain of a patient. One way, as discussed previously, is the careful choice of vector that can transduce CNS cells and that can spread down axons from the site of injection. Another important consideration is the location of the injection site, if you inject into an area with wide spreading projections the vector will be able to travel down these and transduce a larger area of the brain. This has been demonstrated by comparing injection into the striatum to the ventral tegmental area (VTA) in MPS VII mice (Cearley *et al.* 2007). Injections into the VTA resulted in a greater spread of enzyme than injection into the striatum. Another method is to use a convection enhanced delivery system (Bobo *et al.* 1994). This method delivers vector into the brain with positive pressure over a long period of time and is aided by the pulsation of the cerebral arteries (Hadaczek *et al.* 2006). In rhesus monkeys 24µl was infused at 0.2µl/min for 60 minutes followed by 0.4µl/min for 30 minutes and resulted in widespread transduction (Kells *et al.* 2009). Convection enhanced delivery of AAV is well reviewed in Varenika *et al.* (Varenika *et al.* 2009).

1.6.3 *Ex Vivo* Mediated Gene Delivery for MPS Disease

Ex vivo gene therapy is another alternative. This involves the transduction of cells, typically stem cells, outside of the body before re-introduction. This approach commonly uses HSCs and was first demonstrated in 1984 using a murine retroviral vector (Williams *et al.* 1984). It was later demonstrated that the transduced stem cells were able to repopulate the haematopoietic system and that the target gene was expressed *in vivo* (Dick *et al.* 1985; Eglitis *et al.* 1985). This method of delivery was used in the X-SCID gene therapy trials (Hacein-Bey-Abina *et al.* 2003; Cavazzana-Calvo *et al.* 2007). *Ex vivo* gene therapy is well reviewed in (Naldini 2011).

Retrovirally transduced macrophages have been used in the MPS VII mouse model. The intravenously administered macrophages were tolerated, persisted for 38 days and stored GAGs were reduced. This therapy is limited because the macrophages were terminally differentiated, could not migrate to the brain and have a limited lifespan (Ohashi *et al.* 2000). Alternatively, neural stem cells that have been transduced to over express β-glucuronidase, could be transplanted into the brain which should persist as they are not terminally differentiated. This has been evaluated in neonatal MPS VII mice with transduced human neural stem cells. Lysosomal storage was reduced to nearly normal levels after 25 days, however, the number of transplanted cells reduced rapidly. This was thought not to be due to an immune response, as the same occurred in immunodeficient NOD-SCID mice, but was due to apoptotic cell death of the transplanted cells (Meng *et al.* 2003). This approach has also been assessed in Twitcher mice, a model of Krabbe disease (globoid cell leukodystrophy) (Neri *et al.* 2011). These mice were examined 40 days post neonatal transplant and 45-65% of WT enzymatic activity was achieved, which was associated with a reduction in storage of galactolipid in globoid cells and neuroinflammation. An improvement in survival was observed but this was less than has been previously observed with HSCT. Interestingly doubling the injected cell dose had no effect on the number of cells engrafted.

Transduction of HSCs with a lentiviral vector containing IDUA driven by an erythroid promoter successfully limited IDUA expression to erythrocytes in MPS I mice. There was an increase in the enzyme activity in the periphery, some behavioural improvement and in the brain, there was a decrease in vacuolation in cells lining microvessels. Some improvements in the brain can be caused by high circulating enzyme however, it was not clear what the treatment effect was in neurons within the parenchyma of the brain. Therefore it may be better to have enzyme producing cells within the brain past the BBB (Wang *et al.* 2009).

Transplanted stem cells repopulate the haematopoietic system and some of their progeny, monocytes, migrate to the brain and become microglial cells (Priller *et al.* 2001). In the rodent brains the mass of neurons is the same as the mass of non-neuronal cells (microglia and endothelial cells) so microglial cells make up a large

proportion of the brain (Herculano-Houzel *et al.* 2006). In human brains the ratio of neuronal to non-neuronal cells is the same (Azevedo *et al.* 2009; Herculano-Houzel 2009). Microglial cells are able to produce enzyme within the brain, thus cross correcting neuronal cells by providing a source of enzyme to the brain that does not have to pass through the BBB. The first *in vivo* gene therapy experiment for MPS was in MPS VII mice in 1993, when retroviruses were used to transduce HSC to express β-glucuronidase. After sub-lethal irradiation, up to 5% of HSCs were gene modified which was sufficient to decrease storage (Marechal *et al.* 1993). Mild conditioning regimens were also used in conjunction with retrovirus modified HSCs in MPS VI cats, but despite persistence of the transduced cells for 2 years, no clinical effect was observed (Simonaro *et al.* 1999).

A decrease in neurodegeneration was observed in the brains of MPS IIIB mice that had received retrovirus transduced bone marrow (Zheng *et al.* 2004), although the level of gene expression was lower than might be expected using this type of vector. A much more impressive therapeutic effect was observed in a mouse model of the LSD, Metachromatic Leukodystrophy (MLD) and, like MPS IIIA, this disease does not normally respond to HSCT. Lentiviral transduction of HSCs *ex vivo* and transplantation into MLD mice showed a reversal in neurodegeneration (Biffi *et al.* 2006). The source of ARSA, the deficient enzyme in MLD, in the brain was demonstrated to be from microglial cells by immunohistochemistry for HA tagged ARSA. Lentiviral haematopoietic stem cell gene therapy has also been evaluated recently in the mouse model of MPS I. A high level of IDUA activity was achieved with 4.5 times normal activity in the brain, associated with behavioural correction, normalisation of GAGs in the brain and correction of the skeletal phenotype (Visigalli *et al.* 2010). To achieve this, on average 11 copies of the viral vector were integrated to each haematopoietic cell.

Human bone marrow from MPS IH patients has been successfully transduced using a retroviral vector to express significant activity of IDUA that provide long term cross- correction *in vitro*. This proof of principal experiment supports the feasibility of the transduction of human bone marrow to express and secrete lysosomal enzyme (Fairbairn *et al.* 1996). An unsuccessful bone marrow gene therapy trial for

MPS I was conducted in Manchester in 1997-2000 with 3 patients, but cell transduction and gene expression from the retroviral vector was transient, probably because stem cells were not transduced to any significant extent. An immune response to delivered enzyme was also noted in all 3 patients, but no serious adverse effects related to vector delivery were reported (Bigger, personal communication).

The *ex vivo* haematopoietic stem cell gene therapy approach is now being applied in three clinical trials for lysosomal storage disorders. A clinical trial for MPS I is now planned by Dr Alexandra Biffi and will use a lentiviral vector rather than a retroviral vector to transduce the haematopoietic stem cells. This trial will transduce the patient's own bone marrow cells. Additionally, Dr Mark Sands has a gene therapy trial pending in the USA for MPS VII. This clinical trial will attempt to transduce HSCs with a lentiviral vector containing the transgene, GUSB, which is deficient in MPS VII. They will use a non-myeloablative approach and a multiplicity of infection of 10, with two rounds of transduction. Dr Alexandra Biffi has started a clinical trial for MLD using a lentiviral vector and full myeloablative conditioning and autologous CD34+ cells. Three patients have already been enrolled and received transplants with 2 copies per cell achieved in the first patient.

1.7 Lentiviral Mediated Stem Cell Gene Therapy for MPS IIIA

MPS IIIA is currently untreatable, and patients progressively decline to a vegetative state followed by death in the second decade of life (Meyer *et al.* 2007). ERT and other somatic sources of enzyme are ineffective because the neurological phenotype is resistant, due to exclusion of enzyme from the brain by the BBB (Wraith 2006). Central nervous system ERT is a potential therapy that avoids the BBB, however, repeated intracranial injections are invasive and potentially dangerous (Hemsley *et al.* 2008b). Additionally, a humoral immune response against the administered enzyme may reduce its efficacy. SRT has potential, but further optimisation and clinical trials are necessary (Jakobkiewicz-Banecka *et al.* 2007). CNS delivery of gene therapy vector may be a viable approach to treat MPS IIIA if sufficient transduction of the human brain, which is 3500 time heavier than a

mouse brain and 17 times heavier than a macaque, (Herculano-Houzel 2009) can be achieved. Peripheral correction may also be required and there have only been a limited number of trials so far, so the risks are unknown.

We hypothesise that following a standard HSC transplant, insufficient enzyme is produced to effectively treat the disease phenotype. Transducing HSC with an SGSH- lentiviral vector should increase the production SGSH in HSCs to achieve a higher level of SGSH activity than normal HSCT. The transplanted HSCs can repopulate the haematopoietic system and monocytes traffic across the blood brain barrier where they differentiate into microglia cells which will produce enzyme within the brain to treat the neurological phenotype.

1.7.1 Project Aims

1. Develop suitable behavioural measures to determine the effect of therapy on neurodegeneration in the MPS IIIB (Figure 1-10) and MPS IIIA (Figure 1-11) mouse models.
2. Over-express SGSH in HSCs using the SFFV-SGSH lentiviral vector, followed by transplantation into the mouse model of MPS IIIA crossed onto the C57BL/6 background (Figure 1-12).
3. Perform a long-term comparison of lentiviral vectors expressing SGSH in transplanted MPS IIIA HSCs compared to transplants of transduced normal cells and normal cells alone into MPS IIIA mice, to determine the effect on enzyme activity, GAG and HS storage levels, HS sulphation, GM2 ganglioside secondary storage, neuroinflammation and behaviour.

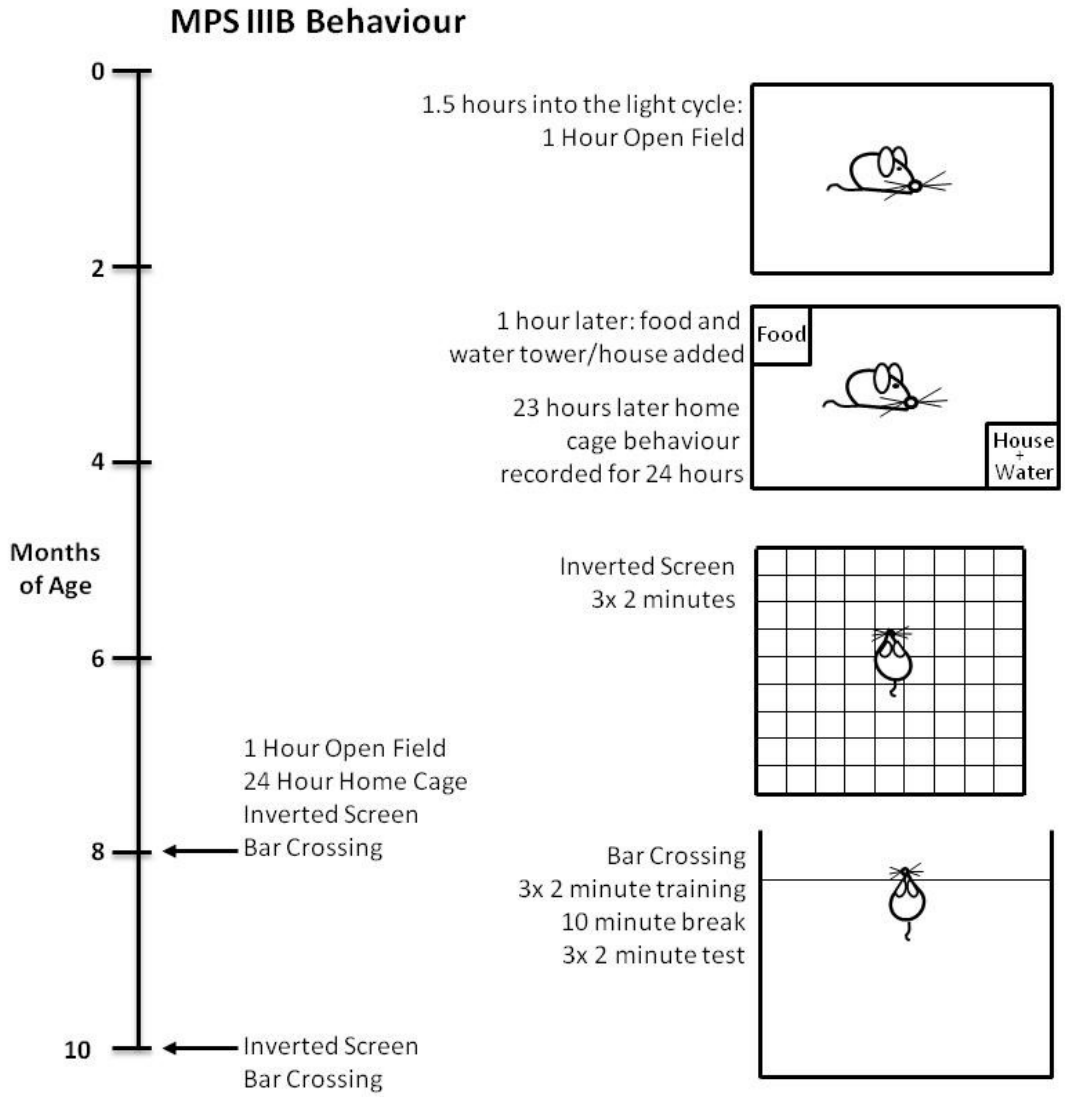


Figure 1-10 Outline of MPS IIIB mouse behavioural testing (Chapter 3)
At 8 months (32 weeks) of age, 1.5 hours into the light cycle WT and MPS IIIB mice were placed in an open field and their behaviour was recorded for 1 hour. Food was then placed into one corner and a water tower that the mice nest underneath was placed in the opposite corner. 23 hours later the behaviour was recorded for 24 hours. At 8 and 10 months of age the inverted screen test was performed 3 times for a maximum of 2 minutes each trial. At 8 and 10 months of age the bar crossing test was performed 3 times for a maximum of 2 minutes in a training phase and 10 minutes later it was performed 3 times in the testing phase. The same mice were tested at 8 and 10 months of age.

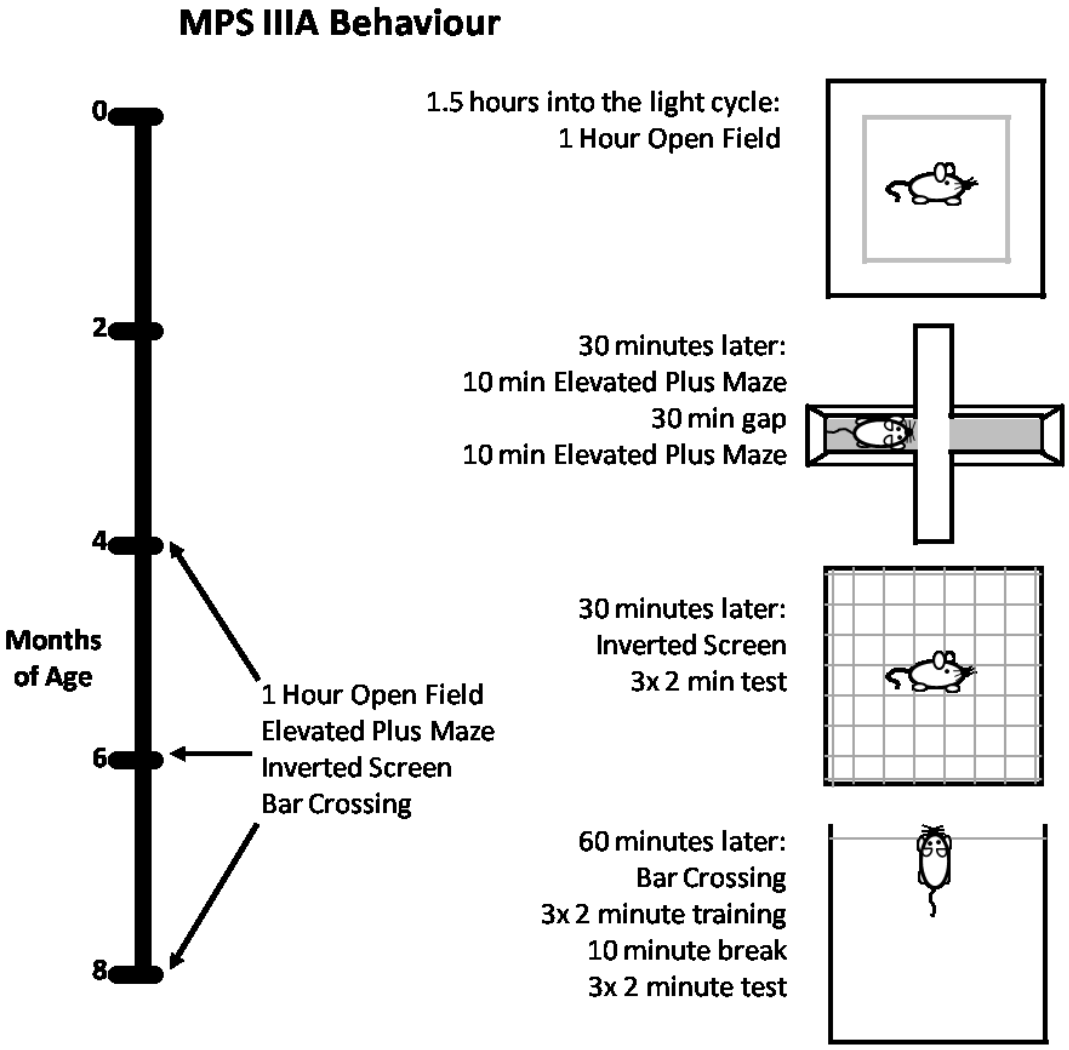
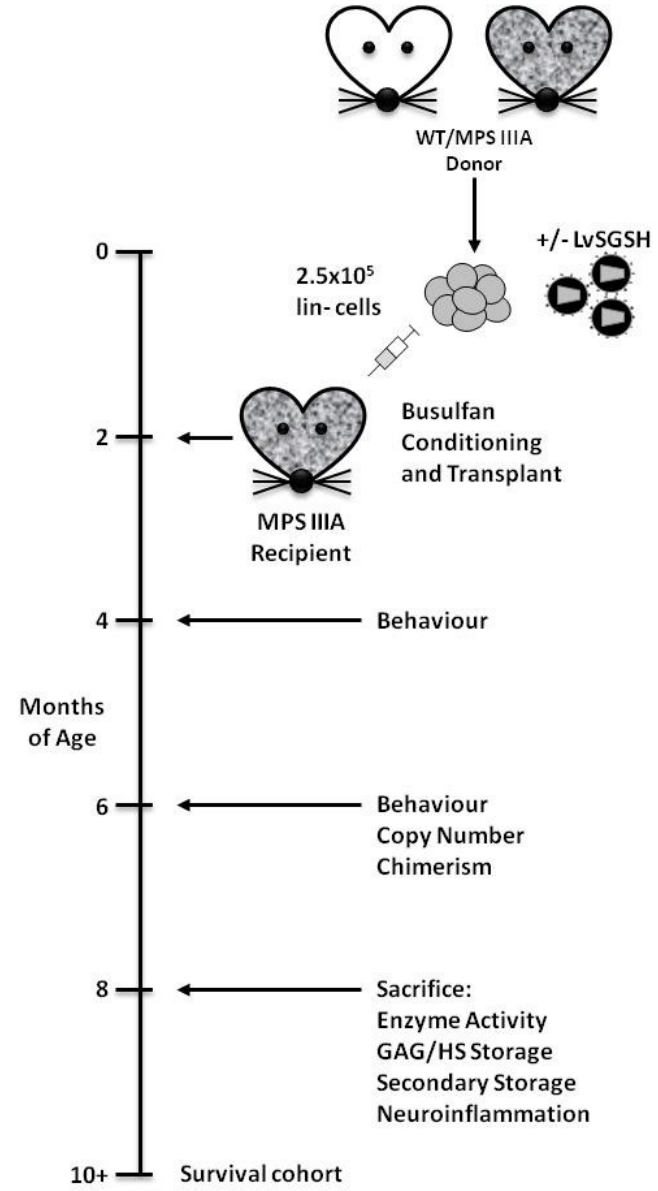


Figure 1-11 Outline of MPS IIIA mouse behavioural testing (Chapter 4)
At 4, 6 and 8 months (16, 24 and 32 weeks) of age, 1.5 hours into the light cycle WT and MPS IIIA mice were placed in an open field and their behaviour was recorded for 1 hour. After a 30 minute break a 10 minute elevated plus maze test was performed. After a 30 minute break the 10 minute elevated plus maze test was repeated. After a 30 minute break the inverted screen test was performed 3 times for a maximum of 2 minutes per test. After a 60 minute break the bar crossing test was performed 3 times for up to 2 minutes in the training period. Then 10 minutes later the test was performed 3 times in the testing period. The same mice were tested at 4, 6 and 8 months of age.



Treatment Groups

Recipient	Donor	Vector	Label	Colour In Chapter 5
WT Control			WT	
MPS IIIA	WT HSCs	+ LVSGSH	LV-WT HSCT	
MPS IIIA	MPS IIIA HSCs	+ LVSGSH	LV-IIIA HSCT	
MPS IIIA	WT HSCs		WT HSCT	
MPS IIIA Control			MPSIIIA	

Figure 1-12 Outline of lentiviral HSC gene therapy for MPS IIIA (Chapter 5)
Bone marrow was removed from donor mice and lineage depleted to leave an enriched HSC stem cell population. HSCs were transduced or mock transduced with a lentiviral vector containing the human SGSH gene and transplanted into myeloablated MPS IIIA recipients. The donor HSCs repopulated the haematopoietic system, produced SGSH and cross-corrected affected MPS IIIA mouse organs. At different time points, various outcome measures were employed to determine the effect of the therapy.

Chapter 2 – Materials and Methods

2.1 Materials

Tissue culture plasticware was sourced from Corning (Amsterdam, The Netherlands) unless otherwise stated. Pipette tips were from StarLab (Milton Keynes, UK). All chemicals were purchased from Sigma Aldrich (Dorset, UK) unless otherwise stated. PCR primers were synthesised by Invitrogen (Life Technologies, Paisley, UK) and QPCR primers and probes were synthesised by Applied Biosystems (Life Technologies, Paisley, UK).

2.2 Maintenance of Mouse Colony

The MPS IIIA mouse model is a spontaneously occurring mouse model caused by a G to A mutation at nucleotide base 91, resulting in a change from aspartic acid to asparagine at position 31 (Bhattacharyya *et al.* 2001). This results in 2-3% residual SGSH enzyme activity. The MPS IIIA mice were obtained on a mixed 129SvJ, C57BL/6, SJL, and CD1 background (Bhaumik *et al.* 1999; Bhattacharyya *et al.* 2001; Crawley *et al.* 2006). The MPS IIIB mouse model was artificially created by targeted disruption of the 6th exon of the *NAGLU* gene with a 900bp insertion (Li *et al.* 1999) and was crossed onto the C57Bl/6 background.

The MPS IIIA and MPS IIIB mouse colonies were maintained by heterozygote x heterozygote breeding to produce wild type litter mates for controls. Heterozygous mice in the MPS IIIB colony were occasionally bred with a C57BL/6J mouse purchased from Harlan to maintain the background. The mice are supplied with standard chow and water *ad libitum*. All experiments were performed with local ethical approval and in accordance with Home Office regulation.

2.2.1 Backcrossing MPS IIIA Mice

The MPS IIIA mice were obtained on a mixed 129SvJ, C57BL/6, SJL, and CD1 background, for reliable and consistent behavioural testing the mice need to be backcrossed on to a pure background (Crawley 2007). Therefore heterozygous mice in the MPS IIIA colony were bred with a C57BL/6J mouse purchased from Harlan, and the heterozygous offspring was then bred with another C57BL/6J mouse purchased from Harlan. This was repeated for 10 generations until the MPS

IIIA mice can be considered to be on the C57BL/6J background (B6.Cg-Sgsh^{mps3a}/6J). The procedure is now occasionally repeated to maintain the background.

An additional colony has also been set up where MPS IIIA mice (B6.Cg-Sgsh^{mps3a}/6J) have been crossed with the PEP3 (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ) mouse strain to produce MPS IIIA mice that express the pan-leukocyte marker CD45.1 rather than CD45.2 (Shen *et al.* 1985). This allows the transplanted cells in MPS IIIA mice to be tracked by flow cytometry. A heterozygous mouse from the MPS IIIA colony was bred with a PEP3 mouse. The offspring all expressed CD45.1 and CD45.2 and were genotyped for heterozygous MPS IIIA status as described in 2.2.3. Heterozygous MPS IIIA mice expressing both CD45.1 and CD45.2 were then bred. The offspring from this cross then had to be genotyped for MPS IIIA status as described 2.2.3 and CD45.1/2 status was determined from a blood sample collected as described in 2.16.1 and genotyped by flow cytometry as described in 2.15. A colony was then established from mice heterozygous for MPS IIIA status who also expressed only CD45.1. The background of this colony was maintained by occasionally backcrossing with a PEP3 mouse.

2.2.2 Isolation of Genomic DNA

DNA was extracted from samples using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma Aldrich) following the manufacturer's instructions. Briefly; tissue was digested in lysis solution T (180μl) with proteinase K (20μl) for 4-6 hours at 55°C, vortexing hourly. Cells were then lysed with lysis solution C (200μl). Ethanol (200μl) was used to precipitate the DNA. The binding column was prepared by loading column preparation solution (500μl) and centrifuging at 12,000g for 1 minute. The DNA was then loaded onto the binding column and centrifuged at 6,500g for 1 minute. Columns were washed twice with wash solution (500μl), first at 6,500g, then at 12000g. The DNA was eluted in Elution Solution (10mM Tris-HCl, 0.5mM EDTA, pH 9.0) (200μl) by centrifuging at 6,500g. DNA concentration was determined using a Nanodrop (Thermo Scientific, Loughborough, UK), elution solution (1.5μl) was pipetted onto the sensor to use as a blank, after reading that it was wiped off and 1.5μl of DNA was pipetted onto the sensor and the DNA concentration was determined from the absorbance by the Nanodrop software.

2.2.3 Genotyping MPS IIIA Mice

The G to A mutation at the 91 position in *SGSH* removes a *Msp*A1I restriction enzyme digestion site therefore the MPS IIIA mice can be genotyped by amplifying DNA by PCR and cleaving it with *Msp*A1I. The size of the DNA fragments can be used to genotype the animals.

MPS IIIA mice were numbered by ear punching using an ear punch (Stoelting, Dublin, Ireland) under isoflurane anaesthesia, and the ear punches were used to genotype the mice. DNA was extracted from the ear punches (see section 2.2), and the *SGSH* DNA was amplified by PCR. The PCR reaction contained: 10x PCR Buffer (5µl, Bioline, London, UK), 0.24mM dNTPs (Invitrogen), 0.5µM forward primer (5' GTGTTCCCTGCCTGCTCAC 3' Invitrogen), 0.5µM reverse primer (5' CCAGTCCCCTCATCCCACTA 3' Invitrogen), 1M Betaine (Invitrogen), 1.5mM $MgCl_2$ (Bioline), 2.5 units Bioline BioPro DNA polymerase, 5µl genomic DNA and ddH_2O up to 50µl. The thermocycling conditions were 96°C for 5 minutes followed by forty cycles of 96°C for 40 sec, 64°C for 40 sec and 72°C for 40 sec; and a final elongation phase at 72°C for 10 minutes.

The DNA (7.8µl) was cut with *Msp*A1I (2 units; New England Biolabs (NEB), Hitchin, UK), in 100µg/ml BSA and NEBuffer 4 (1µl) at 37°C for 1 hour. The DNA fragments were separated by gel electrophoresis (2% agarose (Sigma) in TAE buffer and 1µg/ml EtBr). The genotype can be determined from the pattern of DNA fragments; wild type (WT) SGSH will be digested into 199bp, 118bp and 78bp fragments, the mutant (MUT) into 317bp and 78 bp and the heterozygous (HET) into 317bp, 199bp, 118bp and 78bp.

2.2.4 Genotyping MPS IIIB Mice

The MPS IIIB mouse model was created by targeted disruption of the 6^{th} exon with a 900bp insertion (Li *et al.* 1999). Therefore the size of PCR DNA fragments can be used to genotype the animals.

DNA was extracted from ear punches (see section 2.2.2), and the *NAGLU* DNA was amplified by PCR. The PCR reaction contained: 10x PCR Buffer (5µl, Bioline), 0.24mM dNTPs (Invitrogen), 0.5µM forward primer (5' GGCAACCATGGCCTGTTTGG

3’ Invitrogen), 0.5µM reverse primer (5’ GGCTGACTGGGGTACCTCTTC 3’ Invitrogen), 1M Betaine (Invitrogen), 6mM $MgCl_2$ (Bioline), 2 units Bioline BioPro DNA polymerase, 3µl genomic DNA and ddH_2O up to 50µl. The thermocycling conditions were 96°C for 3 minutes followed by 35 cycles of 96°C for 45 sec, 64°C for 45 sec and 72°C for 3 minutes 30 sec; and a final elongation phase at 72°C for 10 minutes.

The DNA was separated by gel electrophoresis (1% agarose (Sigma) in TAE buffer and 1µg/ml EtBr). Wild type mice have a band at 900 bp, the MPS IIIB have a band at 1800 bp due to the presence of a 900bp insertion and the heterozygous mice have both bands.

2.3 Behavioural Analysis

MPS IIIB mice were analysed at 8 months (32 weeks) of age by open field, followed by the home cage the next day and on a different day inverted screen and horizontal bar crossing. The inverted screen and bar crossing test was also performed at 10 months of age.

The same MPS IIIA mice and treatment groups were analysed at 4, 6 and 8 months (16, 24, 32 weeks) of age. The mice were first placed in the open field test, then after a 30 minute gap the elevated plus maze was performed. Then after a further 30 minute rest the elevated plus maze was repeated. After another 30 minutes rest the inverted screen test was performed and after a 1 hour gap the horizontal bar crossing test was performed.

2.3.1 Open Field

A 1 hour open field test was performed as described previously (Malinowska *et al.* 2010). For the MPS IIIB mice at 8 months of age female mice were dropped into the centre of an arena (260×365mm) with corn cob flooring and recorded with a digital camera (Sony, Tokyo, Japan) for 1 hour. For MPS IIIA a matt white acrylic arena (450mm wide, 450mm deep, 500mm tall) was used and the mice tested at 4, 6 and 8 months old. The open field test was always performed 1.5 hours into the 12 hour light phase in the animal unit. The videos were analysed using Top Scan software (Clever Sys, Reston, USA) for path length, rapid exploratory behaviour, immobility

and frequency, path length, duration and speed in the centre of the cage. Rearing was manually scored by a blinded observer.

2.3.2 Home Cage

After the 1 hour open field test for the MPS IIIB mice, food and a water tower were added to the cage and the mice were allowed to acclimatise for 24 hours before the behaviour was recorded for 24 hours. The behaviour was analysed using the same measures as the 1 hour open field.

2.3.3 Elevated Plus Maze

For the MPS IIIA mice 30 minutes after the open field test, the elevated plus maze test was performed. The mice were placed onto the end of one of the open arms of the elevated plus maze (two 500 mm long, 100 mm wide arms enclosed with 500 mm high sides and two 500 mm long, 100 mm wide open arms 500mm off the floor, lights illuminated the open arms). The behaviour was recorded for 10 minutes and the arena cleaned with 70% IMS, after a 30 minute rest the test was repeated. The behaviour was analysed using Top Scan software to examine the time spent in the open arm and the number of arm entries and the path length.

2.3.4 Inverted Screen

For the MPS IIIA mice, 30 minutes after the elevated plus maze, the inverted screen test was performed (Jeyakumar *et al.* 1999). The mouse was placed on a 470mm square with a 13mm square wire mesh. The screen was rotated over 1-2 seconds through 180°. The rear leg moves and the duration suspended was measured for up to 2 minutes or until the mouse fell off.

2.3.5 Horizontal Bar Crossing

For the MPS IIIA mice, 60 minutes after the inverted screen test, the horizontal bar test was performed (Jeyakumar *et al.* 1999; Malinowska *et al.* 2010). The mouse was allowed to grip the centre of a metal wire 300 mm long and 2mm in diameter, the wire was secured between 2 posts 320mm above a padded surface. The time to reach the side or fall off was recorded up to two minutes. Crossing the bar in x seconds was scored as 240-x, staying on the bar for two minutes was scored as 120 and falling off after y seconds was recorded as the value of y. The test was repeated

three times followed by a 10 minute rest before three tests from which the score was determined.

2.4 Production of hSGSH Lentiviral Vector Transgene Plasmid

To produce lentiviral vector a plasmid containing the transgene and other necessary elements outlined in Figure 2-2 are required. A lentiviral vector backbone containing Att Gateway cloning sites was kindly given to us by Adrian Thrasher and Steven Howe (Demaison *et al.* 2002). Dr Fiona Wilkinson and Dr Bill Bennett performed Gateway cloning (Hartley *et al.* 2000) to insert the human SGSH sequence from an image clone (#5226903, Invitrogen) into the lentiviral vector backbone, see Figure 2-1. The resulting plasmid, pHRsin.SFFV.hSGSH.att.wpre, was used to produce lentiviral vector and the region of this plasmid packaged into the lentiviral vector is outlined in Figure 2-2.

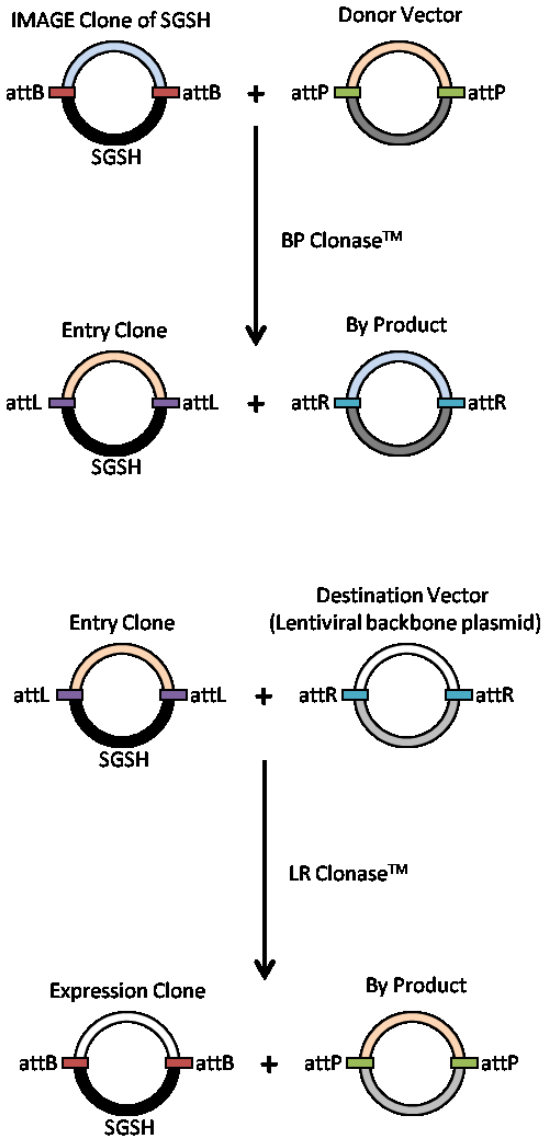


Figure 2-1 Gateway cloning

The IMAGE clone of SGSH was flanked by attB sites, the first stage was therefore to perform the BP reaction to cause site specific recombination of the SGSH sequence into the donor vector backbone and results in the SGSH gene flanked by attL sites. This entry clone can then be added to the destination vector (the lentiviral vector backbone created by Steven Howe) and the LR Clonase reaction recombines SGSH into the lentiviral vector backbone and becomes flanked by attB sites. The plasmid required can be selected for using the different antibiotic resistance genes in the plasmids and by linearising some of the plasmids. Reviewed in (Katzen 2007).

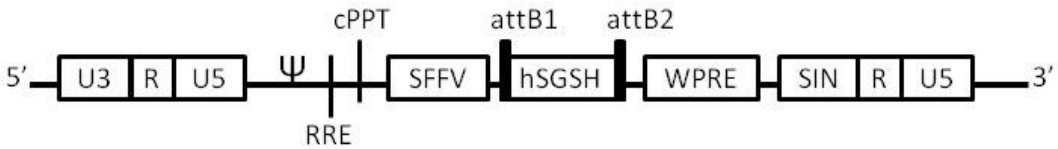


Figure 2-2 Lentiviral vector

Outline of the lentiviral vector, U3, R and U5 are parts of the LTR, Ψ is the packaging signal, RRE is the rev response element, cPPT is the central poly purine tract, att are Gateway clone sites, hSGSH is the human *SGSH* gene, wpre is the woodchuck hepatitis post-transcriptional regulatory element and SIN represents the self inactivating nature of the vector.

2.5 Production of Endotoxin-Free Plasmid DNA

Chemically competent STBL4 bacterial strains (Invitrogen) were transformed with plasmids of interest according to manufacturer's instructions. Colonies on Luria-Bertani (LB) (Invitrogen) agar plates containing 100µg/ml Ampicillin were picked and expanded in LB/Ampicillin. Bacterial plasmid stocks were stored at -80°C in 15% glycerol, in LB broth containing antibiotic.

The bacterial plasmid stock was expanded to produce plasmids for lentiviral production using the Endofree Megaprep kit (Qiagen, Crawley, UK) according to manufacturer's instructions. In brief; bacteria were cultured overnight in LB/ampicillin. One 500ml culture, inoculated with 1ml saturated culture is sufficient for one Qiagen 2500 tip. Bacteria were lysed by alkaline lysis, the plasmid DNA was bound to the Qiagen anion-exchange resin at low pH and salt conditions, washed and eluted. The DNA was precipitated using isopropanol and washed with 70% ethanol. The DNA was resuspended in endotoxin-free TE (1ml; 10mM Tris, 1mM EDTA), filtered through a 0.1µm filter to sterilise, and the DNA concentration was determined using a nanodrop (Thermo Scientific). Typically, 4mg of endotoxin free plasmid DNA was isolated from 1L of bacterial culture.

2.6 Culturing Mammalian Cell Lines

The HeLa cervical cancer and human embryonic kidney (HEK) 293T cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Lonza, Slough, UK), 10% foetal calf serum (FCS) and 2mM L-Glutamine (DMEM/10% FCS) at 37°C with 5% CO_2. Cell lines were passaged at approximately 90% confluency. The media was removed and the adherent cells were washed with phosphate buffered saline (PBS). Trypsin (Lonza; 1ml for a 25cm^2 flask, 2ml for a 75cm^2 flask and 3ml for a 175cm^2 flask) was added and incubated at room temperature (RT) for 5 minutes or until cells were no longer adhered to the flask. Gentle agitation assisted this process. The trypsin was neutralised by the addition of twice the volume of media containing FCS. The cells were centrifuged at 400g for 4 minutes and diluted or counted using a haemocytometer for replating in fresh media. The HeLa cells were typically passaged at 1:8 and the 293T cells at 1:10. The murine lymphoma cell line EL4

(ATCC Number TIB-39, Sigma) was cultured in RPMI 1640 (Lonza), 10% FCS and 2mM L-glutamine. This suspension cell line was passaged by removing cell culture media (0.5 ml) and adding to fresh media (19.5 ml) in a new flask T75 flask.

2.7 Production of High Titre Lentiviral Vector

Lentiviral vector is produced in HEK293T cells by transient transfection with one plasmid containing the viral envelope protein, one with the *gag*, *pol* and *rev* genes and one with the transgene to be packaged as outlined in Figure 1-8. SGSH and enhanced green fluorescent protein (eGFP) lentiviral vectors were produced in batches and concentrated by centrifugation.

On day 1, $6x10^6$ low passage HEK 293T cells were plated per 15cm diameter dish in DMEM (20ml) supplemented with 10% FCS and 2mM L-glutamine. On Day 2 the media was replaced with DMEM/10% FCS. Plates were between 40 and 70% confluent.

Plasmids were transfected using Fugene 6 (Roche, West Sussex, UK) according to manufacturer's instructions. Briefly, transfection solution 1 contained 15µg DNA per plate of genome construct, envelope (pMD2G) and gag/pol 2^{nd} generation (pΔ8.91gag/pol) at a 3:1:2 ratio in H_2O to a total of 18µl/plate. Transfection solution 2 contained 44.5 µl/plate Fugene 6 (Roche) in 1437.5µl Optimem (Gibco, part of Life Technologies) per plate.

Solution 2 was incubated at RT for 5 minutes and added dropwise to solution 1 before incubation for a further 15 minutes at RT. This mixture (1.5ml) was added dropwise to a dish of 293T cells. After 4 hours, the media was carefully replaced with fresh DMEM/10% FCS. 22 or 44 dishes were produced at a time.

The media was changed on day 3. On day 4 and 5 the media was carefully removed from the dishes and transferred to a 225ml graduated conical centrifuge tube (BD, Oxford, UK) on ice and fresh media replaced. The harvested media was centrifuged at 262g (1,110rpm) on a swing out rotor at 4°C for 15 minutes to remove cells and cellular debris. The supernatant was filtered through a 0.45µm low protein binding

cellulose acetate filter (capacity 1L, Nalgene). To concentrate the lentiviral vector the media was transferred to 50ml falcon tubes and centrifuged at 21,191g (13,500rpm) at 4°C for 2.5 hours. The supernatant was removed and the lentiviral vector pellet was resuspended in sterile PBS (50µl per 50ml tube) or formulation buffer (37.5mM NaCl, 19.75M Tris-HCl pH 7, 40mg/ml lactose, 1mg/ml human serum albumin, 5µg/ml protamine sulphate). Lentiviral vector was stored in aliquots of 50µl for use and 2µl for titering at -80°C.

2.8 Titration of Lentiviral Vector

The lentiviral vector from each harvest was titred using HeLa titration for GFP vectors and using EL4 cells and QPCR for SGSH vectors to determine the number of infectious lentiviral vector particles (IU/ml).

2.8.1 HeLa Titration for Infectious Lentiviral Vector Particles

The HeLa cervical cancer cell line (ATCC #CCL-2) was used to determine the infectious titre of the GFP lentiviral vector in this cell type (IU/ml). $1x10^5$ HeLa cells were plated in 6 well plates in DMEM/10% FCS/2mM L-glutamine. After 4 hours at 37°C with 5% CO_2, lentiviral vector was applied to the HeLa cells in triplicate, four dilutions were used 1µl, 0.1µl, 0.05µl and 0.01µl. One set of three wells remained as controls for background auto-fluorescence and one set of three wells was counted to determine the number of cells present at the point of transduction. 24 hours later the media was changed. After a further 24 hours the percentage of GFP expressing cells was determined by FACS analysis on a FACS Canto II Flow Cytometer (BD Biosciences). The cells were washed and incubated with 1ml/well trypsin (Lonza) for 5 minutes at RT or until the cells were detached from the plate; 2ml of DMEM/10% FCS/2mM L-Glutamine was added to neutralise the trypsin. The cells were transferred to FACS tubes, spun down and washed with 2ml FACS buffer (PBS/2% FCS). The cells were resuspended in FACS buffer (200µl) and 100,000 events were collected per tube on the flow cytometer. The infectious lentiviral vector particle (IU/ml) titre was calculated using the following equation.

$$Titre\ \ (IU/ml) = \frac{No\ of\ cells\ at\ transduction\ x\ percentage\ GFP\ positive}{100\ x\ volume\ of\ lentiviral\ vector\ added\ to\ well\ (ml)}$$

The titre can only be accurately measured when the percentage of GFP expression is greater than 0.5% and less than 40% as the majority of GFP positive cells will thus contain only 1 copy of vector per cell.

2.8.2 EL4 Cell Titration for Infection Lentiviral Vector Particles

The EL4 mouse lymphoma cell line was used to determine the number of infections by lentiviral vectors for the GFP and SGSH vectors using QPCR to measure vector integrations rather than mRNA gene expression as this method does not assume that a cell is only transduced once. $1x10^5$ EL4 cells in 100 µl were plated in a 96 well plate in RPMI/10% FCS/2mM L-Glutamine. Then lentiviral vector in five dilutions of 1ml ($1x10^{-3}$ (1µl), $5x10^{-4}$, $1x10^{-4}$, $5x10^{-5}$and $1x10^{-5}$) was applied to the EL4 cells in duplicate. One pair of wells did not have any lentiviral vector added to act as a negative control in the QPCR. The cells were incubated at 37 °C with 5% CO_2 for 24 hours before the media was changed. After a further 48 hours the cells were transferred to 1.5ml Eppendorf tubes and cell pellets frozen. The genomic DNA was then isolated as described in section 2.2.2 and QPCR was performed as described in section 2.8.3 to determine the copy number. The infectious lentiviral vector particle (IU/ml) titre was calculated using the following equation.

$$Titre\ (IU/ml) = \frac{No\ of\ cells\ at\ transduction\ (1x10^5)\ x\ copy\ number}{Volume\ of\ lentiviral\ vector\ added\ to\ well\ (ml)}$$

2.8.3 QPCR for Lentiviral Vector Integration Copy Number

QPCR was used to determine the number of integrations of the lentiviral vector into the genome. The QPCR reaction is based on a probe being cleaved in the polymerisation reaction. This separates a fluorophore from a quencher and the fluorescence emitted is detected after every cycle of the PCR reaction, this is plotted as a change in fluorescence against the cycle number. A standard curve is created using serial dilutions of a sample of known copy number, that is, a house keeping gene, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the sequence of interest.

A fluorescence threshold is determined for each primer and probe set, this is the lowest delta Rn (or fluorescence) that is above background in the linear part of the amplification curve. The threshold cycle (Ct) is plotted in a standard curve against

log “genomes”. (This is a comparative measure between GAPDH endogenous genomes and lentiviral genomes using the probe of interest, therefore an arbitrary number of “genomes” can be assigned to the top value on the standard curve.) The GAPDH and lentiviral probe standard curves are used to determine how many mouse genome copies are present in the sample (GAPDH) relative to the number of lentiviral copies (lentiviral probe). The copy number per diploid cell is the number of lentiviral genomes per 2 GAPDH genomes for each sample. A standard containing a known number of lentiviral integrants per cell is required to generate the standard curve for the lentiviral and GAPDH probes using this approach.

Primers and probe against the murine GAPDH gene (Applied Biosystems) were used to standardise samples as they have a known copy number of 2 per cell. The lentiviral vector integration sites were probed using a primer and probe set against the WPRE element in the lentiviral vector detailed in Table 2-1.

Master mixes for GAPDH and WPRE were made containing 812.5µl of TaqMan Universal PCR Master Mix 2x (Applied Biosystems), 16.25µl of forward primer, 16.25µl reverse primer, 16.25µl probe and 698.75µl of nuclease free water. The final primer and probe concentrations are outlined in Table 2-1. Each master mix was then divided into 24 60µl aliquots, then 2.5µl containing 1-5ng of DNA of each sample was added to the GAPDH and the WPRE master mix. After mixing, 25µl of the master mix plus sample was pipetted in duplicate into 96 well reaction plates (Applied Biosystems) and sealed using an optical plate seal (Applied Biosystems). The reaction was run in an Applied Biosystems 7500 Real Time PCR System, the standard thermocycling conditions were 50°C for 2 minutes, 95°C for 10 minutes and 40 cycles of 95°C of 15 seconds and 60°C for 60 seconds.

Target	Primer/Probe	Concentration	Sequence
GAPDH	Forward primer	100nM	5'-TGCACCACCAACTGCTTAGC-3'(by extrapolation)
	Reverse primer	100nM	5'-AGAACATCATCCCTGCATCC-3' (by extrapolation)
	Probe	200nM	[VIC]-Not released by Applied Biosystems
WPRE	Forward primer	800nM	5'-CCGTTG TCAGGCAACGTG-3'
	Reverse primer	800nM	5'-AGCTGACAGGTGGTGGCAAT-3'
	Probe	200nM	[FAM]5'-TGCTGACGCAACCCCCACTGGT-3' [TAMRA]

Table 2-1 Primer and probe sets for QPCR

2.9 Making a QPCR Standard Cell Line

For accurate copy number determination by QPCR, a known copy number standard is required. The known copy number standard needs to contain the WPRE sequence present in the SGSH lentiviral vector, however we chose to transduce cells with the GFP lentiviral vector that contains the same WPRE sequence because the expression of GFP can be easily assessed using flow cytometry. The known copy number standard also needs to be a clonal cell line so that we can determine the number of copies of WPRE from the number and position of bands by Southern blotting. Therefore we decided to use the EL4 mouse lymphoma cell line (ATCC Number TIB-39, Sigma) as this suspension cell line expands rapidly and exists as a single cell suspension so that when the cells are cloned out by limiting dilution, clumps of cells were not present and colonies are likely to arise from a single cell.

The EL4 cell line was transduced with the pCrimp GFP expressing lentiviral vector at a range of MOI (1, 2, 4, 8, 16 and 32). A sample of the EL4 cells were then analysed by flow cytometry for GFP expression in the FITC channel. The EL4 cells that had been transduced with the lowest MOI that were 95% GFP positive were selected and expanded. After expansion, some of the EL4 GFP positive cells were frozen and others checked by flow cytometry to ensure that they retained GFP expression. The cells were counted by haemocytometer and to confirm that they were in a single cell suspension, and diluted to 10 cells per 100µl. 10 cells (100µl) were pipetted into

the first column of 3x 96 well round bottom plates. Serial dilutions (1:2) were then performed across each plate using a multichannel pipette. The plates were sealed using breathable plate seals (Alpha Laboratories) and were cultured at 37°C, 5% CO_2. After 3-4 weeks the cells furthest to the right of the plate were harvested. These colonies should have arisen from a single cell so they should have the same integration position in every cell. After expanding several different colonies they were assessed for GFP expression by flow cytometry. One GFP positive clone was chosen to be cloned by limiting dilution for a second time. After the second round of cloning by limiting dilution the colonies were assessed again by flow cytometry for GFP expression. All the colonies appeared to express GFP with very similar profile, and mean fluorescent intensity. Therefore it appeared that the two rounds of cloning had succeeded in producing an EL4 GFP expressing cell line from a single cell. The number of copies of WPRE, a sequence within the pCrimp vector that is integrated into the genome was then determined by Southern blot (see section 2.9.1) for 5 of the cell lines to confirm that they all had the same integration pattern and were derived from one cell. DNA from these cells was then used as a standard for QPCR.

2.9.1 Southern Blot for Assessment of Lentiviral Integration Number

2.9.1.1 Genomic DNA Extraction

It is important not to shear the genomic DNA during extraction for Southern blotting as sheared DNA would not run as discrete bands by gel electrophoresis but as a smear. Therefore it would not be possible to quantify the number of integrations of the vector into the genome. Throughout the protocol DNA is never vortexed but stirred with a pipette tip with a sealed end and when pipetted, the pipette tip ends were cut to give a larger bore (3mm). Pipetting was also performed very slowly.

$5x10^7$ EL4 cells were resuspended in 1ml TE (pH 8) and 10ml of lysis buffer was added (TE pH8 + 0.5% SDS + 20µg/ml RNase) for 1 hour at 37°C. Proteinase K was then added to a concentration of 100µg/ml and incubated at 50°C for 3 hours. After leaving to cool to room temperature an equal volume of phenol was added and gently inverted to form an emulsion. The DNA and phenol was then centrifuged at

5,000g for 15 minutes at 4°C. The top aqueous phase was removed and the phenol extraction was repeated. The DNA was precipitated by the addition of 0.2 volumes of 10M ammonium acetate and 2 volumes of 100% ethanol at -80°C for 30 minutes. The precipitated DNA was centrifuged at 5,000g for 5 minutes at RT. The DNA was washed twice with 70% ethanol. The DNA pellet was then air dried for 30 minutes and resuspended in 100µl TE (pH 8) overnight at 4°C. The DNA was quantified using a Nanodrop (Thermo Scientific).

2.9.1.2 Genomic DNA Digestion

The genomic DNA was digested. At this stage it is also important not to shear the DNA so when mixing, a pipette tip with a sealed end was used rather than vortexing or pipetting repeatedly. Additionally all pipetting of the DNA was performed with a wide (3mm) bore pipette tip and very slowly.

25µg of genomic DNA was digested with either 10U of EcoRI-HF (NEB) or 10U of *Bam*HI-HF (NEB) or 5U of *Eco*RI and *Bam*HI in the presence of 10x Buffer 4 and TE up to a total volume of 45µl. The mixture was stirred and the DNA was slowly pipetted into the reaction using a wide bore pipette tip. The reaction was incubated at 37°C for 30 minutes with half the enzyme and then the second half of the enzyme was added and stirred. The reaction was incubated for a further 16 hours.

2.9.1.3 Genomic DNA Transfer

10µg of genomic DNA per lane was then separated by gel electrophoresis (0.7% agarose (Sigma) in TAE buffer and 1µg/ml EtBr) for 7 hours at 40V. The gel was depurinated using 0.125M HCl for 10 minutes with gentle agitation. The gel was then transferred to denaturation buffer (1.5M NaCl, 0.5M NaOH) for 30 minutes with gentle agitation before a final 30 minutes in neutralisation buffer (1.5M NaCl, 0.5M Tris Base, pH 7.5).

The gel was then transferred onto Hybond-XL (GE, Chalfont St Giles, UK) by capillary transfer overnight as per manufacturer's instructions. In brief, a platform was placed in a container and a wick made by placing a sheet of 3MM paper onto the top of the platform and into the transfer buffer (20x SSC; 3M NaCl, 0.3M sodium citrate pH 7). The gel was placed onto the platform, rolled with a pipette to remove

any air bubbles and the rest of the platform covered with plastic. Then a piece of Hybond-XL membrane the same size as the gel was placed on top and rolled with a pipette to remove any air bubbles. Then three pieces of 3MM paper cut to the size of the gel and soaked in transfer buffer were placed without bubbles onto the Hybond-XL. Then 5cm of paper towels were added with a 1kg weight on top and the DNA transferred to the membrane overnight. The next morning the Hybond membrane was placed on blotting paper and baked at 80°C for 30 minutes to fix the DNA before storing at 4°C wrapped in cling film.

2.9.1.4 Producing Southern Blot Probes

40µg of pCrimp plasmid that was used to transduce the EL4 cells was digested with *Bam*HI (NEB) and *Xba*I (NEB) or with *Eco*RI (NEB) and *Eco*NI (NEB) with buffer 4 at 37°C for 1 hour. The digested DNA was then mixed with 6x loading buffer (Sigma) and separated by gel electrophoreses on a 1% agarose gel. For the *Bam*HI and XbaI digest the 751bp fragment was gel extracted from a 983bp and 7.5kb DNA fragment. For the EcoRI and EcoNI digest the 1kb fragment was isolated from the 2kb and 6kb fragments.

Gel extraction was carried out as per manufacturer's instructions (QIAquick, QIAGEN). In brief 1 volume of agarose gel containing the band of interest was incubated with 3 volumes of buffer QG at 50°C for 10 minutes whilst shaking. After the gel had dissolved, 1 volume of isopropanol was added and the DNA was bound to the silica membrane in a column in the presence of a high salt buffer with a pH of less than 7.5. Impurities were washed out using 0.5ml buffer QC and 0.75ml buffer PE containing ethanol. The DNA were then eluted in low salt and higher pH conditions using 30µl of TE (pH 8.5).

From this template DNA a radioactive probe was produced using Ready-To-Go DNA Labelling Beads (GE Healthcare) and radioactive P^{32} dCTP. The manufacturer's method was followed. In brief, 25-50ng of probe DNA was denatured by heating to 95°C for 3 minutes and then placing on ice for 2 minutes. The denatured DNA (in less than 45µl) was then added to 5µl of P^{32} dCTP (3,000 Ci/mmol) and water up to 50µl in the ready-to-go tubes and mixed. The reaction was incubated for 5-30 minutes at 37°C. During this time oligolabelling occurs, random

oligodeoxyribonucleotides present in the tube bind to the template DNA and act as primers for DNA synthesis by DNA polymerase with free nucleotides, including the radiolabeled P^{32} dCTP.

After incubating at 37°C the hybridisation probe was purified from the unincorporated dCTP with a illustra NICK column (GE). The probe DNA was loaded onto a washed Sephadex G-50 column and allowed to pass though by gravity. The probe was then eluted by addition of buffer and gravity flow. However the unincorporated dCTP remains within the beads as the smallest molecules are eluted last as they are able to pass though the pores into the beads.

2.9.1.5 Probe Hybridisation

The probe was hybridised to the Hybond membrane in hybridisation tubes in a hybridisation oven. The Hybond-XL (GE) manufacturer's protocol was followed. The membrane was pre-wet in water, the pre-warmed to 65-68°C Denhardt's buffer (5x SSC (0.75M NaCl, 0.075M sodium citrate pH7), 5x Denhardt's solution (2% BSA, 2% Ficoll 400, 2% Polyvinylpyrrolidone) and 0.5% SDS). The membrane was then added to a hybridisation tube and 30-70µl/cm^2 of Denhardt's buffer added. The probe produced in 2.9.1.4 was then denatured by heating to 100°C for 5 minutes before placing on ice for 3 minutes. 0.5-2x10^6 counts per ml of probe Denhardt's buffer was added in prehybridisation buffer (5x SSC, 5x Denhardt's solution, 0.5% SDS, 40mg/L sonicated salmon sperm DNA), not directly onto the membrane. The blot was then hybridised overnight at 65°C. The blot was then rinsed with 2 x SSC 0.1% SDS, then washed twice for 5 minutes with 2x SSC/0.1% SDS, then twice for 10 minutes with 1x SSC/0.1% SDS and finally four times for 5 minutes with 0.1x SSC/ 0.1% SDS. The blot was then wrapped in cling film and Hyperfilm MP was exposed for 24 hours at -80°C (Figure 2-3).

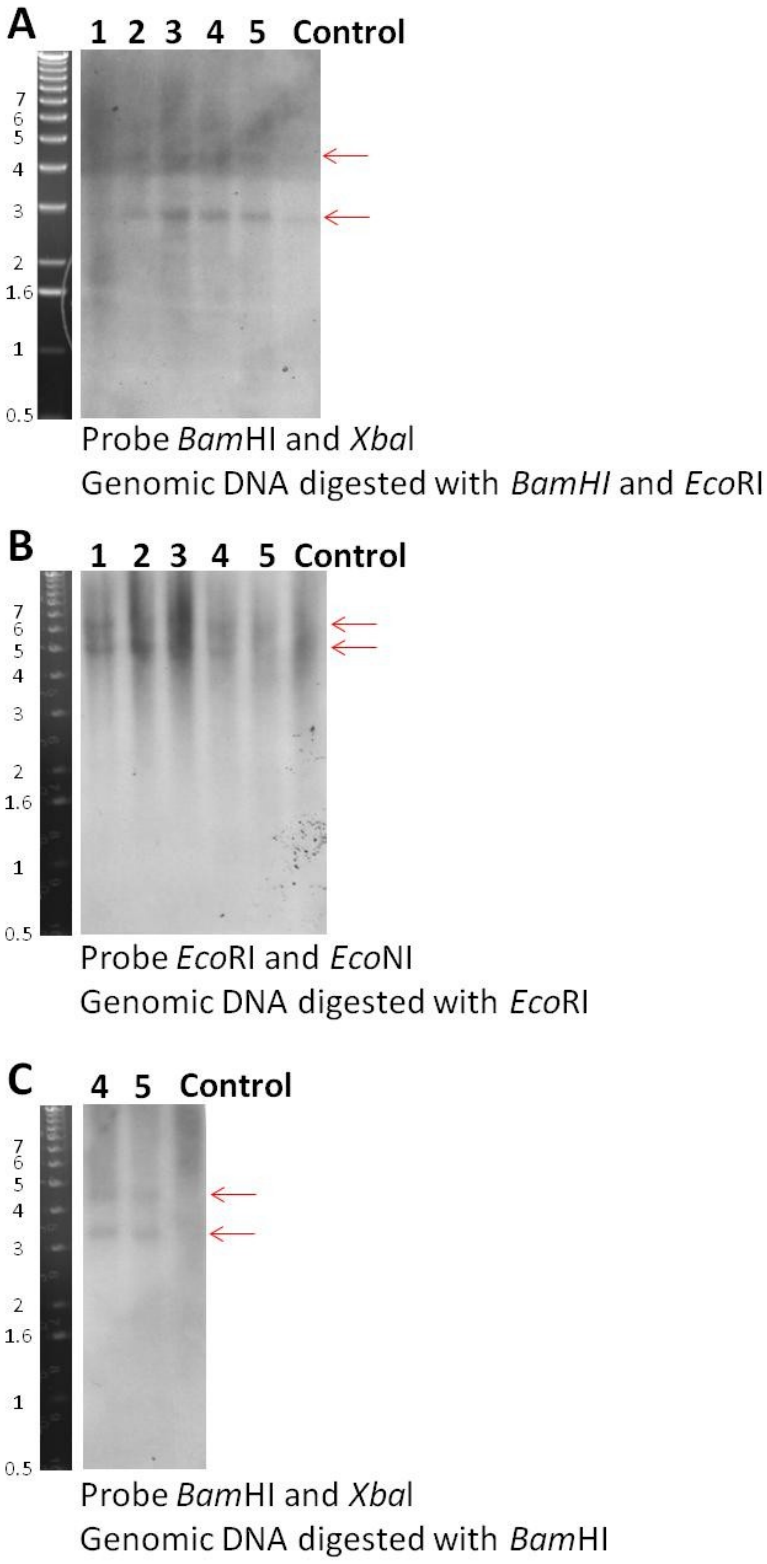


Figure 2-3 Southern blot

Five EL4 GFP clones (1-5) from the second round of cloning by limiting dilution and untransduced EL4 cells (Control) were analysed by Southern blotting. A 1Kb ladder (Invitrogen) shows the size in Kb down the left hand side. (A) The genomic DNA was cut with *Bam*HI and *Eco*RI and the probe was made using *Bam*HI and *Xba*I. (B) The genomic DNA was cut with *Eco*RI and the probe was made using *Eco*R1 and *Eco*NI. (C**)** The genomic DNA was cut with *Bam*HI and the probe was made using *Bam*HI and *Xba*I. There are consistently two bands in each of the clones (1-5) and no bands in the control. This indicates that two copies of the vector have integrated per cell.

2.10 Bone Marrow Isolation

The donor mice were culled and sprayed with 70% ethanol to kill any bacteria, they were then skinned and the legs removed and placed into PBS pen/strep to kill bacteria. The femur, tibia, and ilium (pelvis) bones were removed from the flesh by scraping with a number 22 scalpel (Appleton Woods, Birmingham, UK). A 29G insulin syringe was used to flush bone marrow into PBS/2% FCS and cells were kept cold on ice throughout the procedure. Tissue debris was removed using a 70µm filter (Cell Trics). 5-6 volumes of ammonium chloride lysis buffer pH 7.2-7.4 (150mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA,) was applied to the bone marrow to lyse red blood cells. After 10 minutes incubation on ice, the lysis was stopped by the addition of 1 volume FCS. The cells were then washed twice with PBS 2% FCS.

2.11 Haematopoietic Stem Cell Enrichment

The haematopoietic stem cell population of murine bone marrow was enriched by the removal of progenitor cells using StemSep mouse progenitor enrichment kit (Stemcell Technologies, Grenoble, France) following the manufacturer's instructions with minor modifications to conserve antibody. In brief, bone marrow cells at a concentration of $1x10^8$ cells/ml in PBS/2% FCS (twice the recommended concentration) were incubated with normal rat serum at 4°C for 15 minutes to block non-specific binding of antibody. They were incubated (4°C for 15 minutes) with StemSep Enrichment Cocktail containing biotinylated monoclonal antibodies to the following cells or cell surface antigens: CD5, TER119, CD45R, Ly-6G, CD11b and 7-4. After washing with PBS/2% FCS, the cells were incubated at 4°C with anti-biotin tetrameric antibody complexes for 15 minutes followed by magnetic colloid for 15 minutes. A 0.6" column was placed in the magnet and was primed for use by running room temperature PBS at 0.6 ml/min (setting 3 on a Minipuls 3 Peristaltic Pump, Gilson) up the column making sure there were no air bubbles and then 3 column volumes of PBS/2% FCS down the column at 2ml/min (setting 10). The cells were then applied to the column and run at 2ml/min, the column was never allowed to run dry and extra PBS/2% FCS was added 1ml at a time for the first 5ml. Cells without lineage markers were able to pass through and 25ml was collected

and centrifuged at 300g for 10 minutes to concentrate them. Cells were resuspended in a suitable volume of X-VIVO-10 medium (see section 2.12).

2.12 Transduction of the Enriched Haematopoietic Stem Cells

HSCs were cultured in X-VIVO-10 medium (BioWhittaker, part of Lonza) without serum supplementation, containing murine SCF (Peprotech, London, UK) and mFlt3-Ligand (R&D Technologies, Abingdon, UK), both at 50ng/ml, and mIL-6 (Peprotech) at 10ng/ml. The cells were cultured in 24-well plates at a concentration of 1.0 x 10^6 cells per well in 1ml media. After 1 hour at 37 °C with 5% CO_2, lentiviral vector was applied to the cells at a multiplicity of infection (MOI) of 30-60 and incubated overnight.

2.13 Transplantation

Recipient mice were myeloablated with 125mg/kg Busulfan (Busilvex; Pierre Fabre, Toulouse, France) over 5 days. Mice were manually restrained and a 25mg/kg doses were delivered using an insulin syringe (BD) into the peritoneal cavity every day for 5 days in a total volume of 200µl made up with PBS. The recipients were 6-10 weeks old and received acidified water, irradiated food, mash and sugar free jelly during Busulfan treatment and for 6 weeks post BMT to prevent gastrointestinal infections and to encourage fluid uptake. Mice were warmed for 10 minutes in their cage on a hot block to dilate the blood vessels in the tail. The mouse was then placed into a restraining tube (Harvard Apparatus Limited, UK) and 1-2.5x10^5 transduced or untransduced lin- enriched HSCs were administered in 200µl PBS via tail vein injection using an insulin syringe (BD).

2.14 Flow Cytometry

Flow cytometry was carried out on a FACS Canto II Flow Cytometer (BD Biosciences) using FACSDiva (BD Biosciences) analysis software. Events were separated based on forward and side scatter and the cell population was gated from this to exclude

cellular debris from the analysis. From this cell population the live cells were gated by staining with ToPro3 Iodide (Invitrogen) in the APC channel (excitation (ex) 595, 633, 635, 647, emission (em) 660nm) and selecting the unstained cells. Cells without any ToPro3 Iodide staining were used to determine the position of the negative peak and therefore where the gate should be located. For all other antibody stains used in sections 2.8.1 and 2.15 an antibody isotype control for each fluorophore was used to determine the position of the negative peak. Automatic compensation was also performed to account for overlap of fluorescence between channels (FITC ex 488 em 519nm, PE ex 488, 532 em 578 and APC) on the flow cytometer. 100,000 cells were collected for each sample.

2.15 Determination of Chimerism After HSC Transplantation

Donors and recipients were distinguished by measurement of the pan-leukocyte markers CD45.1 and CD45.2 in the blood, bone marrow or spleen. Blood samples (100µl) were taken via a tail vein bleed, and sodium citrate (50µl of 4% w/v, Sigma) was added to prevent clotting (see 2.16.1). After lysing the red blood cells using ammonium chloride lysis buffer for 10 minutes on ice twice, the cells were washed twice in PBS/2% FCS. The cells were incubated with PBS/2% FCS/5% mouse serum at 4°C for 15 minutes to inhibit non-specific binding and were stained with CD45.1 (PE, excitation 488, 532 emission 578nm) and CD45.2 (FITC, excitation 488nm, emission 519) antibodies diluted 1:50 (BD Biosciences) for 30 minutes at 4°C. The cells were washed and resuspended in 200µl PBS/2% FCS. ToPro3 iodide was used to stain dead cells immediately before analysis of the cells on a BD FACS Canto II flow cytometer.

2.16 Sample Collection From *In Vivo* Experiments

2.16.1 Blood Sample Collection

Blood was taken by tail vein bleed for detecting chimerism and to isolate plasma and peripheral blood mononucleocytes (PBMCs). Mice were warmed for 10 minutes in their cage on a hot block to dilate the blood vessels in the tail. The

mouse was then placed into a restraining tube (Harvard Apparatus Limited, UK) and a small cut was made to the tail vein using a number 15 scalpel blade (Appleton Woods). 100µl of blood was collected with 50µl of 4% (w/v) sodium citrate to avoid clotting, then centrifuged at 200g for 10 minutes and the plasma supernatant removed and frozen in aliquots. The remaining cells were lysed twice with ammonium chloride lysis buffer on ice for 10 minutes as per section 2.11. The cells were centrifuged at 200g for 10 minutes and the cell pellet frozen at -80°C.

2.16.2 Perfusion

At sacrifice, mice were perfused with Tyrode's solution (138mM NaCl, 1.8mM $CaCl_2$, 0.3mM NaH_2PO_4, 5.6mM glucose, 12mM $NaHCO_3$ and 2.7mM KCl) heated to 37°C to flush blood from the organs. This allows clearer histological analysis of the brain for lymphoid infiltration without masking from circulating cells and clear biochemical data as the brain tissue is measured not the brain and blood. Mice were anaesthetised with Hypnorm/Hypnovel and the heart exposed. A needle was inserted in the left ventricle and the Tyrode's solution was gently injected. A cut was made above the right atrium. As the heart beats, Tyrode's solution is pumped around the body and the blood is pumped out of the cut above the right atrium. The blood is displaced by the Tyrode's solution and the organs become noticeably paler.

2.16.3 Tissue Processing

After perfusion the brain was removed and separated into two hemispheres. One hemisphere was fixed in 4% paraformaldehyde (PFA/PBS) and the other was snap frozen. The brain was fixed for 24 hours and perfused with 30% sucrose 2mM $MgCl_2$/PBS for 48 hours before freezing at –80°C. The spleen was removed and cut into four segments, three of which were snap frozen. The liver was also removed and three small pieces from the outside and the inside were snap frozen. A sample of spleen and liver was fixed in 4% PFA/PBS for 48 hours followed by 70% ethanol for 48 hours. Liver and spleen samples were progressively dehydrated, cleared in xylene and embedded in paraffin wax in a Microm tissue processor (Thermo Fisher).

2.17 Quantification of Total Protein

The total protein concentration of tissue samples was determined using a BCA assay kit (Thermo Scientific) to standardise tissue samples for the SGSH and Blyscan assays. The assay was performed following the manufacturer's instructions. In brief, a standard curve was constructed using decreasing concentrations of albumin from 2mg/ml to 0.2mg/ml. Samples of tissue were homogenised using a pestle and mortar followed by sonication to lyse the cells. This was performed in homogenisation buffer (0.5M NaCl, 0.02M Tris pH 7-7.5) (200µl for liver and spleen, 100µl for brain). Samples were diluted to ensure that they were within the standard range; plasma, monocytes, spleen and brain were diluted 1:35 and liver was diluted 1:50. 10µl of each standard and sample was loaded in triplicate in a 96 well plate and BCA reagent (200µl per well) was applied. The plate was sealed and incubated at 37°C for 30 minutes. The absorbance at 562nm was recorded and the protein concentration determined using the standard curve.

2.18 SGSH Enzyme Activity Assay

SGSH sulphatase activity is determined using a two step protocol with a fluorescent substrate (Karpova *et al.* 1996). In the first reaction SGSH desulphates the substrate and in the second reaction α-glucosidase hydrolyses the substrate and releases a fluorescent product. 20mM 4-methylumbelliferyl-α-D-N-sulphoglucosaminide.Na (MU-αGlcNS; Moscerdam, Oegstgeest, The Netherlands) and protease inhibitor (1.9mM Pefabloc; Roche Diagnostics) in substrate buffer (143mM Na-barbital, 143mM Na-acetate buffer, 0.7% NaCl, 0.02% (w/v) Na-azide, pH 6.5) are incubated with 40µg of protein for monocytes, plasma, liver and spleen or 60µg of protein for brain in 10µl at 47°C for 17 hours. Samples were run in duplicate in a black 96 well plate (Nunc, Thermo Fischer). The desulphation reaction was stopped with double concentrated McIlvaine's solution Pi/Ci (0.4M $NaHPO_4$, 0.2M citric acid, 0.02% NaN_3, pH 6.7). 10U/ml α-glucosidase (10µl/well) was added to hydrolyse and release the fluorescent 4-Methylumbelliferyl (4MU) in an incubation of 24 hours at 37°C. The reaction was stopped with 0.5M $NaHCO_3$/0.5M Na_2CO_3/0.025% (w/v) Triton X-100 pH 10.7. A standard curve was constructed from 4MU of standard

concentration (66.67µM) diluted in stop buffer and the fluorescence at excitation 360nm and emission 460nm was measured on a Synergy HT Microplate Reader (BioTek, Bedfordshire, UK).

2.19 Blyscan Assay for Total Glycosaminoglycans

The total amount of sulphated glycosaminoglycans were analysed using the Blyscan assay kit (Biocolor, Carrickfergus, UK). Samples of tissue were homogenised using a pestle and mortar followed by sonication to lyse the cells. This was performed in homogenisation buffer (0.5M NaCl, 0.02M Tris pH7-7.5) using 200µl for liver and 100µl for brain. Protein concentration was then determined using the BCA assay (Section 2.17). To prepare samples 20µg of actinase E was added to 100µl of standard or tissue supernatant containing 100µg of protein. The tissue was incubated at 55°C for 20 hours and then the temperature was raised to 100°C for 5 minutes. After cooling the sample was centrifuged at 3,000g for 10 minutes. The sample supernatant was then incubated with 1,9-dimethymethylene blue for 30 minutes whilst shaking. The samples were centrifuged at 10,000g for 15 minutes at 4°C. The pellet of GAGs was incubated with dye dissociation reagent for 15 minutes at room temperature before reading absorbance using a spectrophotometer at 656nm. The quantity of sulphated GAGs was calculated using a standard curve of known concentration samples.

2.20 AMAC for Heparan Sulphate

AMAC analysis of heparan sulphate (HS) in brain tissue was performed by Dr Rebecca Holley as described in (Deakin *et al.* 2008; Holley *et al.* 2011). Firstly the HS was extracted from one tenth of a brain by homogenisation in 10ml PBS using a Dounce homogeniser and treated with 1mg Pronase (100µg/ml) for 4 hours at 37°C followed by addition of 100µl Triton X-100 (1% v/v) for 2 hours at RT and a final Pronase treatment with an additional 1mg of Pronase for 4 hours at 37°C. HS was isolated by passing the digested tissue through a pre-equilibrated 1cm DEAE-Sephacel column and washing with 50ml of 0.25M NaCl, 30mM NaP, pH 7. The HS

was then eluted with 5ml of 1.5M NaCl/20mM NaH_2PO_4 and desalted using a pre-packed PD10 column (GE Healthcare). 1ml aliquots of the eluted HS were loaded onto the PD10 column and allowed to run to waste, before washing with 2ml of water and running to waste. A second 2ml of water was then added, and the eluant was collected (HS fraction), before the column was washed with 20ml of water to remove the salt, ready for the next 1ml aliquot of sample to be added. The resultant heparan sulphate in water was then freeze dried. The HS was then digested with heparinase I, II and III (Seikagaku) at a concentration of 5mIU of each in 100µl of 0.1M NaC_2H_3O and 0.1M $Ca(C_2H_3O_2)_2$ pH7 overnight and freeze dried for a second time.

The resulting HS disaccharide units were dissolved in 10µl of 0.1M 2-aminoacridone (AMAC) in 85% Me_2SO 15% CH_3COOH for 20 minutes at RT. 10µl of 1M $NaBH_3(CN)$ was then added and incubated for 16 hours at RT. The fluorescently AMAC-labelled HS disaccharides were then separated by reverse phase high performance liquid chromatography (RP-HPLC) on a Zorbax Eclipse XDB-C18 RP-HPLC column (3.5µm, 2.1mm × 150mm Agilent Technologies, Edinburgh, UK) equilibrated with 0.1M ammonium acetate. After a 2ml gradient of 0-10% methanol the HS disaccharides were eluted in 50ml of a 10-30% methanol solution at 1ml/min. Disaccharides were detected by in-line fluorescence (excitation at 425nm and emission at 520nm). Disaccharides were identified by comparison with known disaccharide standards (Iduron, Manchester, UK), following labelling with AMAC and RP-HPLC, as described above. Relative quantification of the percentage contribution of each disaccharide species was determined following integration of fluorescent peak area and application of an AMAC labeling efficiency correction factor to account for the different amount of labeling of the different HS forms as described previously (Deakin *et al.* 2008; Holley *et al.* 2011). Total heparan sulphate levels were compared by summing the total fluorescent units.

2.21 Immunohistochemistry

2.21.1 Preparation of Mouse Brains for Free Floating Immunohistochemistry

The snap frozen hemisphere of brain processed in 2.16 was cut using a Zeiss Hyrax S30 microtome. The cerebellum of the brain was removed and the rest mounted in OCT (Raymond A Lamb, part of Thermo Fisher) on the microtome and the 30µm sections were collected into 3x 96 well plates containing TBSAF (15% sucrose, 30% ethylene glycol, 0.05% NaN_3 in TBS) and stored at 4°C.

2.21.2 GM2 Gangliosides

Secondary storage of GM2 gangliosides was assessed using an antibody against GM2 gangliosides. 4 equally spaced sections of brain from 0.26 to -1.94mm relative to bregma were placed in a 24 well plate and the endogenous peroxidase activity was blocked with 600µl of PBS/1% H_2O_2 for 30 minutes. The sections were washed for 4x 5 minutes with PBS + 0.01% saponin. Non-specific background staining was blocked with PBS + 1% bovine serum albumin (BSA) /10% normal goat serum (Vector Labs) and 0.03% saponin for 1 hour. The sections were then washed for 4x 5 minutes with PBS/0.01% saponin.

The primary antibody (a gift from Dr Dobrenis and Prof Walkley) was diluted 1:40 in PBS with 1% normal goat serum, 1% BSA and 0.02% saponin overnight at 4°C. The sections were washed for 4x 5 minutes with PBS/0.01% saponin. The sections were incubated with a biotinylated goat anti mouse IgM secondary antibody (Vector Labs) for one hour diluted 1:200 in PBS with 1% normal goat serum, 1% BSA and 0.02% saponin. The sections were then washed for 4x 5 minutes with PBS/0.01% saponin and incubated with avidin/biotin (Vector Labs) (5µl solution A + 5µl solution B per ml of PBS/0.01% saponin) for 60 minutes. The sections were then washed for 4x 5 minutes with PBS and incubated with DAB substrate containing nickel (Vector SK-4100) for 5 minutes. The reaction was stopped with ice cold PBS and washed twice with PBS before mounting onto positively charged slides (Thermo Scientific Superfrost Plus).

The slides were left overnight to dry and then rehydrated in TBS for 5 minutes before progressively dehydrating them with increasing concentrations of ethanol

followed by xylene. The cover slips were mounted using DPX (Merck, Nottingham, UK).

Four sections per mouse, with an n of 5 mice per group, were examined under a light microscope (Axiovision, Carl Zeiss, Welwyn Garden City, UK). From each of the 4 sections the same two regions of the cortex were photographed at the same time and under the same setting for all mice. The images were quantified using Image J software to quantify the level of black GM2 ganglioside staining and the background staining was deducted.

2.21.3 Isolectin B4 for Microglial Cells

Neuroinflammation was assessed by staining with isolectin B4 for microglial cells. 4 equally spaced sections of brain from 0.26 to -1.94mm relative to bregma were placed in a 24 well plate and the endogenous peroxidase activity was blocked with 600µl of TBS 1% H_2O_2 for 30 minutes. The sections were then washed with TBS, 2mM $MgCl_2$, 1mM $CaCl_2$ for 5 minutes 3 times. The sections were then incubated with isolectin B4, a lectin from *Bandeiraea simplicifolia* conjugated to peroxidase (BSI-B4; Sigma) diluted 1:200 overnight at 4°C in TBS/$MgCl_2$/$CaCl_2$. The sections were washed for 4x 5 minutes with TBS/$MgCl_2$/$CaCl_2$ and incubated with DAB substrate (Vector SK-4100) for 22 seconds. The reaction was stopped with ice cold TBS and washed twice with TBS before mounting onto positively charged slides (Thermo Scientific Superfrost Plus).

The slides were left overnight to dry and then rehydrated in TBS for 5 minutes before staining with Mayer's haematoxylin for 5 minutes. The sections were rinsed in PBS pH 8 until it run clear and then rinsed in water. The sections were progressively dehydrated with increasing concentrations of ethanol followed by xylene. The cover slips were added using DPX (Merck).

The number of activated microglial cells was quantified from two fields of view of cortex on four sections from each mouse.

2.21.4 LAMP2 for Lysosomal Compartment

A section from -0.84mm relative to bregma from 2 mice per group was stained for LAMP2, a lysosomal membrane protein, to demonstrate the size of the lysosomal

compartment. Sections were placed in a blocking solution of 5% goat serum, 1mg/ml BSA and 0.1% Triton X-100 in TBS and incubated at room temperature for 1 hour to stop non-specific binding. The sections were then stained with LAMP2 IgG (2µg/ml) in blocking solution, a control section was also stained with rat IgG at the same concentration as the LAMP2 antibody. The sections were incubated overnight at 4°C and washed for 4x 5 minutes with TBS followed by staining with Alexa 488 goat anti-rat (green) 1:1000 in blocking solution and incubated for 1 hour at room temperature in the dark to avoid photo bleaching. After washing once with TBS the sections were incubated with 300nM DAPI for 15 minutes at room temperature to stain all nuclei. After 4x 5 minute washes with TBS the sections were mounted on positively charged slides (Thermo Scientific Superfrost Plus) and after excess TBS was removed, ProLong Gold Anti-fade mounting medium (Invitrogen) was added, followed by the cover slip. The slides were then kept in the dark until examination by confocal microscopy. Representative regions of the cortex have been shown.

2.22 Statistical Analysis

Data were analysed by Student's t-test, by one way ANOVA or two way ANOVA with Tukey post hoc test and by MANOVA using JMP version 8 (SAS Institute Inc, Cary, NC, USA) where applicable. The *F* value is given for each comparison, together with degrees of freedom and the probability of error in Chapter 3. Where data failed normality tests it was log transformed so that it was normally distributed. Significance was assumed for probabilities of 0.05 or lower. Survival analysis was performed in SPSS version 19 (IBM, New York, US) using Kaplan-Meier analysis with Mantel-Cox log rank pairwise comparisons.

Chapter 3 - MPS IIIB Behaviour

Hyperactive Behaviour in the Mouse Model of Mucopolysaccharidosis IIIB in the Open Field and Home Cage Environments

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and the published version can be found in the appendix.

3.1 Abstract

Mucopolysaccharidosis IIIB (MPS IIIB) is a lysosomal storage disorder characterised by severe behavioural disturbances and progressive loss of cognitive and motor function. There is no effective treatment, but behavioural testing is a valuable tool to assess neurodegeneration and the effect of novel therapies in mouse models of disease.

Several groups have evaluated behaviour in this model, but the data are inconsistent, often conflicting with patient natural history. We hypothesise that this discrepancy could be due to differences in open field habituation and home cage behaviour. Eight month old wild type and MPS IIIB mice were tested in a 1 hour open field test, performed 1.5 hours after lights on, and a 24 hour home cage behaviour test performed after 24 hours of acclimatisation. In the 1 hour test, MPS IIIB mice were hyperactive, with increased rapid exploratory behaviour and reduced immobility time. No differences in anxiety were seen. Over the course of the test, differences became more pronounced with maximal effects at 1 hour. The 24 hour home cage test was less reliable. There was evidence of increased hyperactivity in MPS IIIB mice, however, immobility was also increased, suggesting a level of inconsistency in this test.

Performance of open field analysis within 1-2 hours after lights on is probably critical to achieving maximal success as MPS IIIB mice have a peak in activity around this time. The open field test effectively identifies hyperactive behaviour in MPS IIIB mice and is a significant tool for evaluating effects of therapy on neurodegeneration.

3.2 Introduction

Mucopolysaccharidosis type IIIB (MPS IIIB, OMIM #252920), or Sanfilippo syndrome, is caused by a deficiency in the lysosomal enzyme, α-N-acetylglucosaminidase as a result of mutations in the *NAGLU* gene (O'Brien 1972; Zhao *et al.* 1996). NAGLU deficiency impairs lysosomal degradation of heparan sulphate in the brain, resulting in enlarged lysosomes, secondary storage of gangliosides and cholesterol (Constantopoulos *et al.* 1978; McGlynn *et al.* 2004), severe neuroinflammation (Ohmi *et al.* 2003; Canal *et al.* 2010) and progressive cellular and organ dysfunction (Valstar *et al.* 2008). Patients present with severe behavioural problems including aggression, hyperactivity, a decreased sense of danger and sleep disturbances (Cleary *et al.* 1993; Fraser *et al.* 2005; Moog *et al.* 2007) leading to progressive neurodegeneration and death in the second decade of life, although the phenotype varies considerably (Meyer *et al.* 2007; Heron *et al.* 2011).

There is currently no effective therapy for MPS IIIB, but substrate reduction therapy (Malinowska *et al.* 2009; Malinowska *et al.* 2010), enzyme replacement therapy (Yu *et al.* 2000), and gene therapy and stem cell therapy approaches (reviewed in Valstar *et al.*, 2008) have been evaluated. Surrogate clinical biomarkers for MPS IIIB have only recently been forthcoming and are still in development (Langford-Smith *et al.* 2010; Langford-Smith *et al.* 2011c) therefore the behavioural phenotype of the mouse model remains an important tool for measurement of treatment effect on the severe neurodegenerative phenotype seen in both mice and patients.

The mouse model of MPS IIIB has previously been reported to demonstrate alterations in circadian rhythms as measured in the home cage, including increased activity in the light (Heldermon *et al.* 2007; Canal *et al.* 2010) or dark (Cressant *et al.* 2004). Interestingly, none of these studies, including our own, demonstrated overall increases in activity of MPS IIIB mice. In contrast, an 8 minute open field analysis of the MPS IIIB mouse model, half in light, half in dark, showed reductions in activity in both light and dark phases (Li *et al.* 1999), whilst a separate study observed reduced rearing in the second 30 minutes of a 1 hour open field test (Fu *et al.* 2007). This

contrasts with observations of hyperactivity in MPS IIIB mice in a 10 minute open field test (Cressant *et al.* 2004).

MPS IIIB mice also demonstrate a reduced sense of danger in some situations. Fear conditioning was normal in the contextual fear test but reduced in tone fear test (Li *et al.* 1999) which could be caused by differences in sensory function, whilst no changes in the acoustic startle response have been observed (Fu *et al.* 2007). A reduced sense of danger (increased entry to open arms) has been observed in the elevated plus maze test when performed in the dark (Cressant *et al.* 2004) but not in the light (Fu *et al.* 2007). Therefore, again it is not clear if the MPS IIIB mice mimic the pathology seen in patients where they have a reduced sense of danger.

These disparities may reflect different testing conditions, and require definitive characterisation in both the home cage and open field to determine a reliable phenotype.

3.3 Methods

3.3.1 Mouse Maintenance

MPS IIIB mice (Li *et al.* 1999) were maintained by heterozygous breeding as described (Canal *et al.* 2010) on a C57BL/6J background with occasional backcrossing to the C57BL/6J line (Harlan, UK) to maintain the background. WT and MPS IIIB KO female littermates were used for all behavioural analysis. All procedures were ethically approved and in accordance with the UK Home Office and European Union (2010/63/EU) regulations. Mice had access *ad libitum* to food and water and were housed under a 12 hour light-dark cycle.

3.3.2 Behavioural Testing

The 1 hour open field test was performed as described previously (Malinowska *et al.* 2009; Malinowska *et al.* 2010). In brief, 8 month old female mice (n=6 WT, n=6 MPS IIIB) were dropped into the centre of an arena (260×365mm) containing corn cob flooring and the behaviour was recorded with a digital camera (Sony) for 60 minutes. The open field test was performed 1.5 hours after the lights were turned

on in a randomised blinded fashion. The behaviour was analysed using Top Scan software (Clever Sys. Inc. Reston, USA). The path length, rapid exploratory behaviour (speed >90mm/s), immobility (speed <0.05mm/s) and frequency, path length, duration and speed in the central 25% of the cage were analysed.

Rearing was manually counted by an observer blinded to the genotype of the mice. The number of supported rears (using the side of the cage) and unsupported rears were counted for the first 10 minutes of the 1 hour open field.

In the 24 hour home cage test a mouse (n=6 WT, n=7 MPS IIIB) was placed in a cage (260×365mm) with food and a plastic water tower where water is accessible and the mice can nest underneath (Figure 3.3A). Behaviour in this area could not be analysed as it is hidden from view. The mice were allowed to acclimatise for 24 hours before 24 hours of filming. When the lights were turned off, infra red lights automatically turned on to illuminate the mice for the night-vision enabled camera. Behaviour was analysed as for the 1 hour open field using the same measures and Top Scan Suite software (Cleversys Inc, Reston, VA, USA).

The inverted screen test was performed at 8 months (n=4 WT, n=5 MPS IIIB) and 10 months (n=3 WT, n=4 MPS IIIB) of age by placing the mouse on a 470mm square screen covered with a 13mm square wire mesh and rotating the screen through 180° over 1-2 seconds. The mouse was suspended upside down above a padded surface and the number of rear leg moves was counted and the amount of time spent suspended up to a maximum of 2 minutes was recorded.

The horizontal bar test was performed as described previously (Malinowska *et al.* 2010) at 8 months (n=6 WT, n=7 MPS IIIB) and 10 months (n=3 WT, n=4 MPS IIIB) of age. In brief a 300mm metal wire 2mm in diameter was secured between 2 posts 320mm above a padded surface. The mouse was allowed to grip the centre of the wire and the time to fall or reach the side was recorded, after two minutes the test was stopped. Crossing the bar in x seconds was scored as 240-x, remaining on the bar was scored as 120 and falling off the bar after y seconds was recorded as the value of y. The test was repeated three times as a practice run followed by a 10 minute rest prior to three tests where the score was recorded.

3.3.3 Statistical Analysis

Data were analysed using JMP software (SAS Institute Inc, Cary, NC, USA) and MANOVA repeated measure analysis and one way ANOVAs were used, where appropriate, to determine differences between groups (Genotype) and within groups (Illumination). The *F* value is given for each comparison, together with degrees of freedom and the probability of error. Significance is assumed at probabilities of less than 0.05.

3.4 Results

In order to test the hypothesis that MPS IIIB mice, like patients, are more hyperactive and have a reduced sense of danger, groups of 6-7, 8 month old female wild type (WT) and MPS IIIB mice were monitored for 1 hour for habituation behaviour in the open field. In order to check the reliability of the home cage test, following 24 hours of acclimatisation, we monitored the same mice in the home cage environment for a further 24 hours. We also performed tests of motor function at 8 months and 10 months of age to monitor motor decline.

To compare the open field test (Figure 3-1A) to previous studies and to determine the minimum time required to achieve consistent significance, we have presented the data as per minute activity graphs and analysed the first 3 and 10 minutes of the test as well as the total 60 minutes for significance, using a student's t test (Cressant *et al.* 2004; Hemsley *et al.* 2005). MPS IIIB mice travel more than 3 times as far as WT mice over the duration of the trial (Figure 3-1B) with significant differences observed in the first 3 ($F_{1,10}$=13.63, p=0.004), 10 ($F_{1,10}$=19.69, p=0.001) and 60 minutes ($F_{1,10}$=20.70, p=0.001) of the trial.

Rapid exploratory behaviour was monitored by the frequency (Figure 3-1C) and duration (Figure 3-1D) of speed over 90mm/s. Highly significant increases in both of these parameters were observed in MPS IIIB mice at 3, 10 and 60 minutes after commencement of the test ($F_{1,10}$=17.98–33.11, p=0.002-p=0.0002). The supplementary video (supplementary video 3-1) shows the median mouse from each group over the median 2 minutes of the open field test (minutes 29-31), running at 4 times normal speed, in which many of these behaviours can be observed.

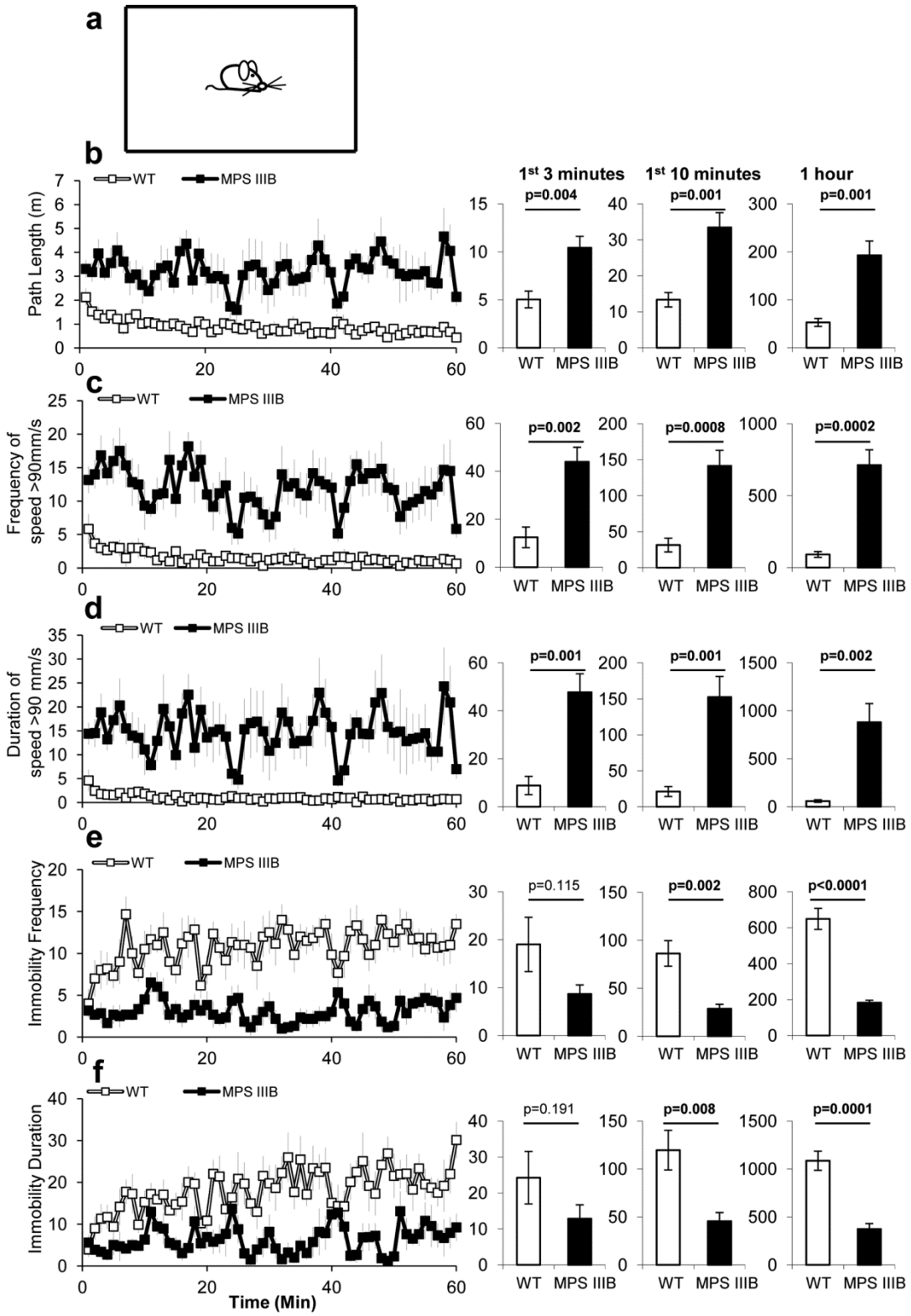

Figure 3-1 One hour open field – activity

At 8 months of age 6 WT (white squares) and 6 MPS IIIB (black squares) mice were placed in the centre of a cage 260 x 365mm outlined in (A) and the behaviour was recorded for 1 hour. The results of the open field behaviour are presented as a 1 hour period with the average of every minute presented and as a bar chart of the first 3 minutes, first 10 minutes and whole hour. Error bars represent the standard error of the mean (SEM). *p* values were calculated by t-test. The mean average path length in meters (B), frequency of rapid exploratory behaviour (speed> 90mm/s) (C), duration of rapid exploratory behaviour (speed> 90mm/s) (D), frequency of immobility (E) and duration of immobility (F) are presented.

We also monitored frequency (Figure 3-1E) and duration (Figure 3-1F) of immobility over the period of the test. MPS IIIB mice had a consistently reduced frequency and duration of immobility but this was only significant when measured at 10 and 60 minutes ($F_{1,10}$=16.67–60.24 p=0.008- p<0.0001) and was not significant at 3 minutes ($F_{1,10}$=2.99–1.97, p=0.11–p=0.19). In these cases, the groups became more significantly different as the analysis period was extended. Interestingly, this appears to be due to an initial similarity in the behaviour of the two groups when the mice were first placed in the cage, that rapidly diverges over time (Figure 3-1E,F).

As a prey species, mice tend to display thigmotaxis (Simon *et al.* 1994), and thus show a reduced tendency to enter the central part of an open field arena. In Figure 3-2A the frequency of entries into the centre of the open field was recorded every minute for 1 hour. Figure 3-2B shows the path length in the central area and shows a very similar pattern. In the first minute, the WT and MPS IIIB mice are indistinguishable, but diverge in subsequent minutes with the MPS IIIB mice entering the centre more frequently and travelling further. This results in no significant differences between WT and MPS IIIB mice in the first 3 minutes ($F_{1,10}$=4.63–2.33, p=0.57–0.16) but a significant increase in MPS IIIB centre entries and path length after 10 minutes ($F_{1,10}$=8.29–9.04, p=0.02-0.01) and a greater significant difference after 60 minutes ($F_{1,10}$=21.19–23.45, p=0.001-0.0007). MPS IIIB travel at significantly higher speeds in the central area (Figure 3-2C) with improving significance from 3 ($F_{1,10}$=13.52, p=0.0043) to 10 ($F_{1,10}$=22.50, p=0.0008) to 60 minutes ($F_{1,10}$=78.65, p=0.000005). This parameter is consistent with the overall rapid exploratory behaviour and may not be related to a reduced sense of danger. Figure 3-2D shows that there is no clear difference in the duration spent in the centre of the open field, after 3 minutes there is no significant difference ($F_{1,10}$=2.58, p=0.140), at 10 minutes the WT mice spend significantly more time in the centre ($F_{1,10}$=10.03, p=0.010); however after 60 minutes there is no significant difference ($F_{1,10}$=1.48, p=0.251). Both groups of mice spent significantly more time in the sides of the test area than in the centre (not shown) ($F_{1,10}$=296.36, p=9x10^{-9}), thus they both display a tendency to thigmotaxis. Overall these data suggest that although MPS IIIB mice enter the central area more frequently and travel further

and more rapidly in this area, there is no significant reduction in the sense of danger in MPS IIIB mice in this test in terms of the duration spent within the central area.

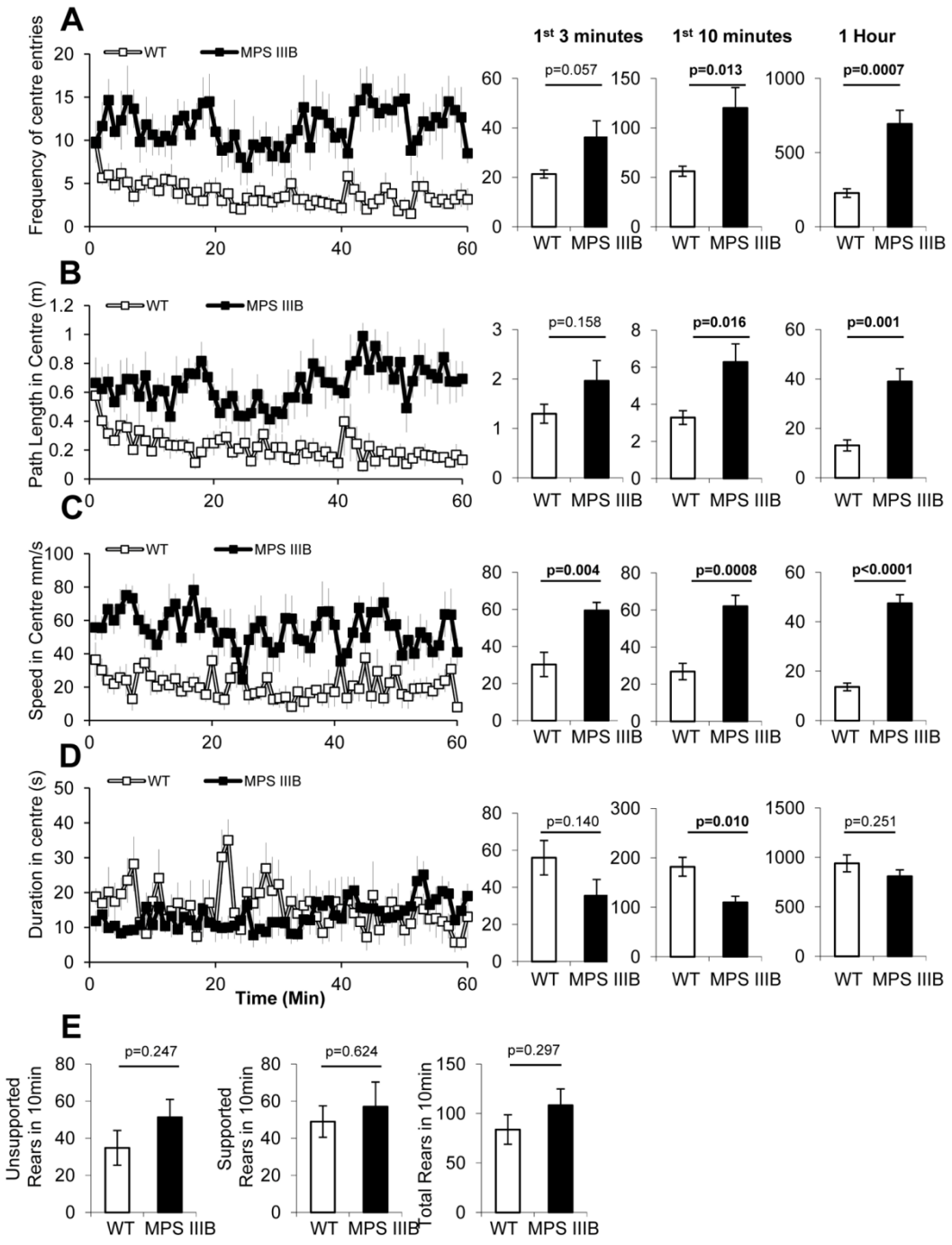

Figure 3-2 One hour open field – centre and rearing

At 8 months of age 6 WT (white squares) and 6 MPS IIIB (black squares) mice were placed in a cage outlined in Figure 3-1A and the behaviour was recorded for 1 hour. The results of the open field behaviour are presented as a 1 hour period with the average of every minute presented and as a bar chart of the first 3 minutes, first 10 minutes and whole hour. Error bars represent the SEM. *p* values were calculated by t-test. The frequency of entering the centre (A), path length in the centre of the cage in meters (B), the speed in the centre in mm per second (C) and time spent in the centre in seconds (D), the frequency of rearing (E) are presented.

Finally, we measured rearing activity of mice over the first 10 minutes of the open field test (Figure 3-2E). This is not measurable using our automated system and was therefore scored by hand. No significant difference was identified in the number of unsupported rears ($F_{1,10}$=1.51, p=0.247), supported rears using the side of the cage ($F_{1,10}$=0.256, p=0.624) and total rears ($F_{1,10}$=1.21, p=0.297) between MPS IIIB or WT mice.

The same groups of female mice were acclimatised in the same test arena as the open field for 24 hours by supplying bedding, a house, food and water *ad libitum* (Figure 3-3A). Home cage behaviour was then monitored for the subsequent 24 hours.

Figure 3-3B shows the average path length travelled by groups of 6-7 WT or MPS IIIB mice in 20 minute intervals over 24 hours, split into two equal 12 hour light or dark periods. All mice have significantly increased path length in darkness than in light (Illumination; $F_{1,11}$=15.46 p=0.002) and over 24 hours, MPS IIIB mice have a significant increase in path length over their WT counterparts, travelling on average 656m compared to 298m for WT mice (Genotype; $F_{1,11}$=5.29, p=0.03). However, there are no significant differences between MPS IIIB and WT when measured in only darkness (Genotype; $F_{1,11}$=2.56, p=0.138) or light (Genotype; $F_{1,11}$=3.22, p=0.100). This suggests that a minimum of 24 hours observation in the home cage is required to show significant differences in path length.

Rapid exploratory behaviour is measured as frequency (Figure 3-3C) and duration (Figure 3-3D) of speed over 90mm/s as before. All mice have an increased frequency of speed over 90mm/s in darkness compared to light (Illumination; $F_{1,11}$=9.23, p=0.013). MPS IIIB mice have a borderline significantly increased frequency of speed over 90mm/s over the entire 24 hour period (Genotype; $F_{1,11}$=4.05, p=0.057) and an almost significant difference between genotypes when this is measured in light (Genotype; $F_{1,11}$=4.27, p=0.063) but not in darkness (Genotype; $F_{1,11}$=2.48, p=0.144) alone, due to the increased variability in the dark. Interestingly the duration of speed over 90mm/s showed that MPS IIIB mice spent significantly less time travelling faster than 90mm/s over 24 hours (Genotype; $F_{1,11}$=7.91, p=0.01) and in the dark (Genotype; $F_{1,11}$=8.80, p=0.01) which is not

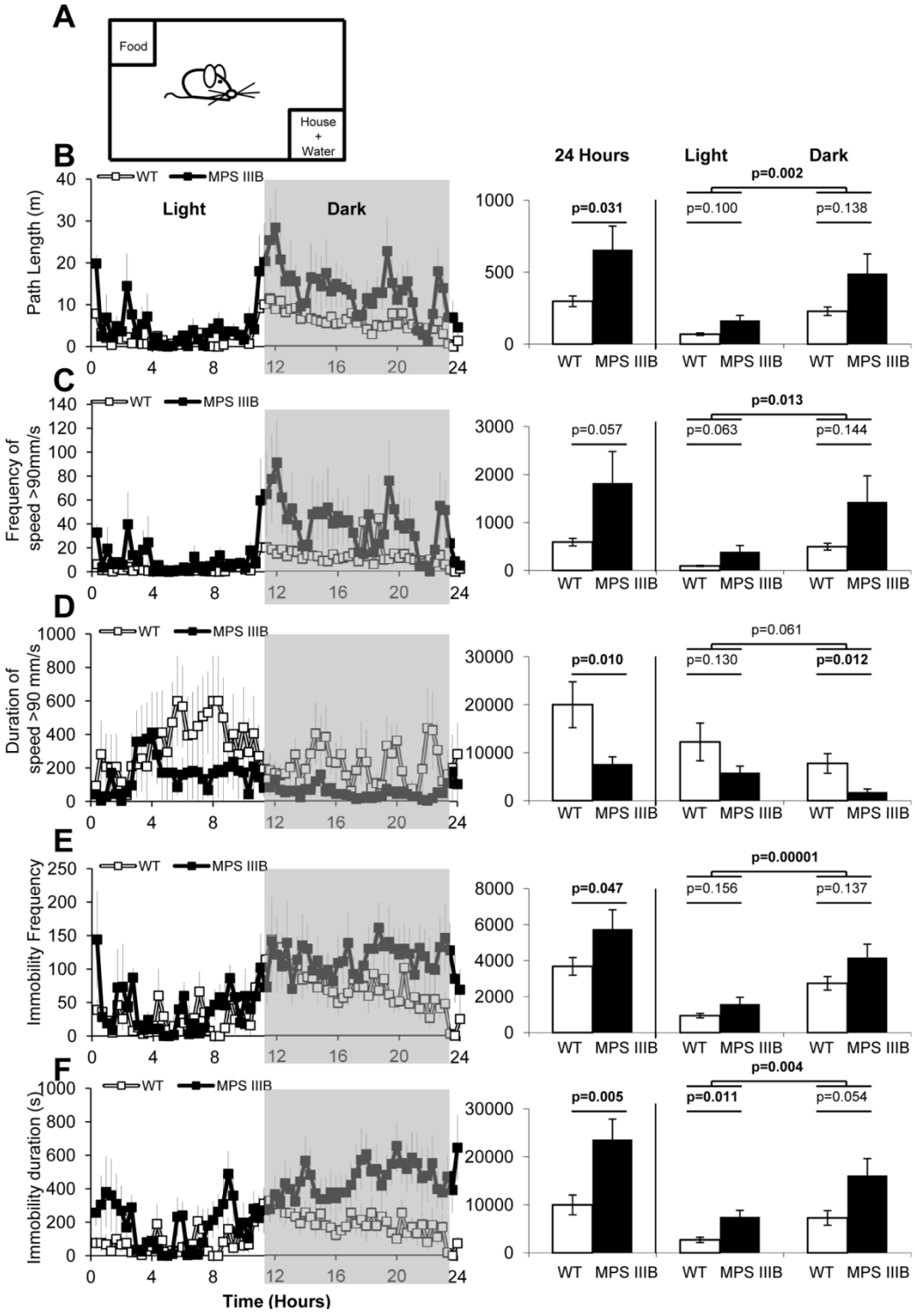

Figure 3-3 Home cage behaviour – activity

At 8 months of age 6 WT (white squares) and 7 MPS IIIB (black squares) mice were placed in a cage 260 x 365mm outlined in (A) that included a food bowl and nesting house containing water within it. After 24 hours of acclimatisation the home cage behaviour was recorded for 24 hours using infrared lights for night-time monitoring. The results of the home cage behaviour are presented as a 24 hour period with the average of every twenty minutes presented and as a bar chart of firstly the whole 24 hours and secondly the light and dark periods separately. Error bars represent the SEM. *p* values were calculated by MANOVA repeated measure analysis and one way ANOVA. The mean average path length in meters (B), frequency of rapid exploratory behaviour (speed> 90mm/s) (C), duration of rapid exploratory behaviour in seconds (D), frequency of immobility (E) and duration of immobility (F) are presented.

consistent with the frequency, pathlength nor the results of the 1 hour open field test.

The frequency (Figure 3-3E) and duration (Figure 3-3F) of immobility were also recorded over 24 hours. Both the frequency (Illumination; $F_{1,11}$=54.24, p=0.00001) and duration (Illumination; $F_{1,11}$=9.32, p=0.004) of immobility was significantly increased in darkness for all mice and overall MPS IIIB mice had increased frequency (Genotype; $F_{1,11}$=4.42, p=0.047) and duration (Genotype; $F_{1,11}$=9.69, p=0.005) of immobility. For duration of immobility we observed a significant genotype effect in light (Genotype; $F_{1,11}$=9.32, p=0.011) and dark (Genotype; $F_{1,11}$=4.67, p=0.054).

In the 24 hour home cage test the overall frequency of entering the centre (Figure 3-4A) was significantly higher in the dark (Illumination; $F_{1,11}$=17.46, p=0.0015) and there was an overall increase in centre entries by MPS IIIB mice (Genotype; $F_{1,11}$=6.96, p=0.015). In light there is also a significant effect (Genotype; $F_{1,11}$=6.52, p=0.027). This parameter is consistent with the hyperactivity observed in the 24 hour home cage test and 1 hour test. The distance travelled in the centre (Figure 3-4B) is also consistent with hyperactivity as there is a significant overall increase in MPS IIIB path length in the centre (Genotype; $F_{1,11}$=6.14, p=0.021) and in the light (Genotype; $F_{1,11}$=5.21, p=0.043) but not in the dark (Genotype; $F_{1,11}$=2.98, p=0.112). There is also increased activity for all mice in the dark compared to light (Illumination; $F_{1,11}$=7.10, p=0.017). The speed in the centre is likewise consistent with hyperactivity with a significant overall genotype (Genotype; $F_{1,11}$=5.71, p=0.026) and illumination effect (Illumination; $F_{1,11}$=8.31, p=0.015). However the increased speed in the centre observed in MPS IIIB mice only occurs in the light (Genotype; $F_{1,11}$=40.99, p=0.00005) and not in the dark (Genotype; $F_{1,11}$=0.16, p=0.69). There is a significant illumination difference in the duration of time spent in the centre (Figure 3-4D) (Illumination; $F_{1,11}$=12.69, p=0.0045) but no genotype difference. This suggests that there is no detectable difference in sense of danger between MPS IIIB and WT mice.

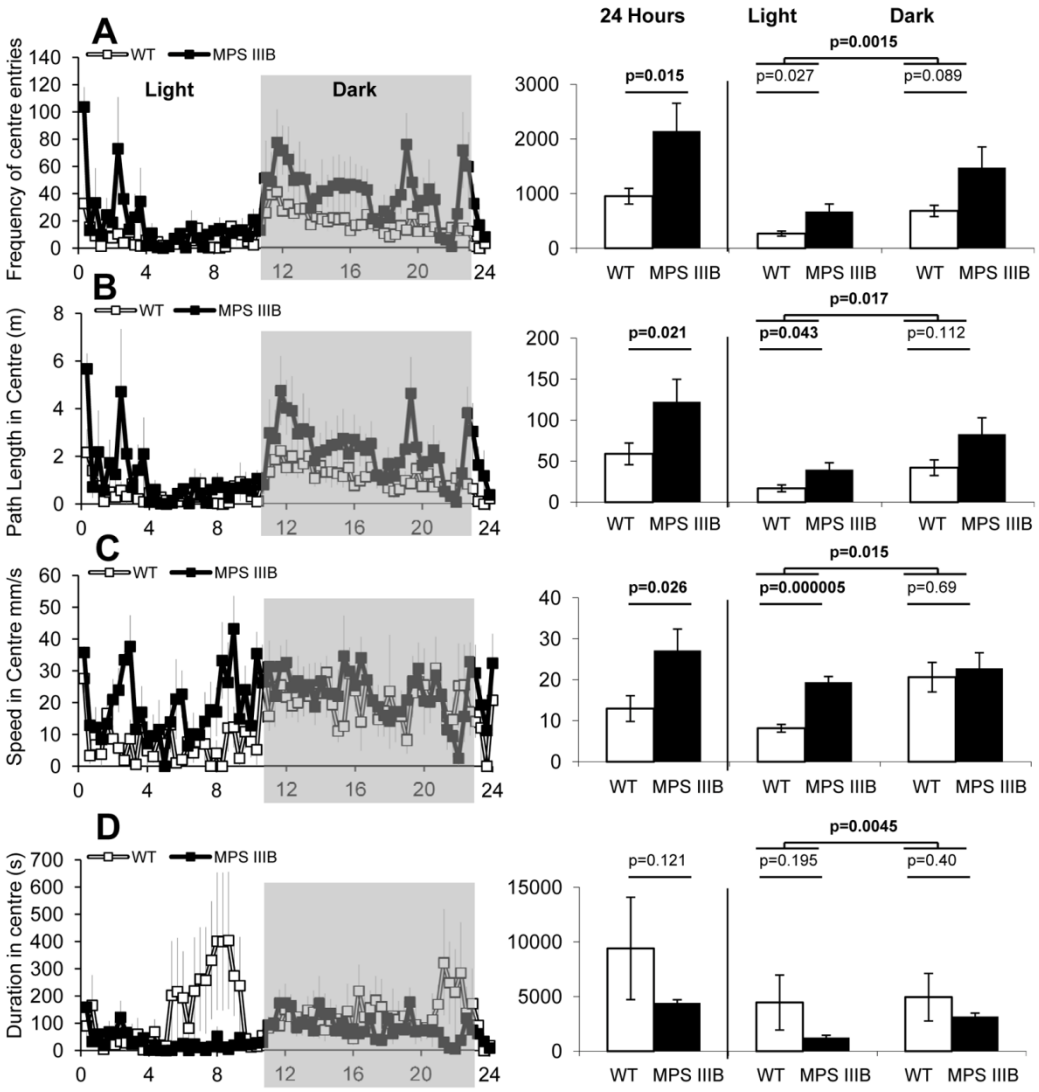

Figure 3-4 Home cage behaviour – centre
At 8 months of age 6 WT (white squares) and 7 MPS IIIB (black squares) mice were placed in a cage outlined in Figure 3-3A, after 24 hours of acclimatisation the home cage behaviour was recorded for 24 hours. The results of the home cage behaviour are presented as a 24 hour period with the average of every twenty minutes presented and as a bar chart of firstly the whole 24 hours and secondly the light and dark periods separately. Error bars represent the SEM. *p* values were calculated by MANOVA repeated measure analysis and one way ANOVA. The frequency of entering the centre (A), path length in the centre of the cage in meters (B), the speed in the centre in mm per second (C) and time spent in the centre in seconds (D) are presented.

In order to assess loss of motor skills and muscle strength with time, we firstly used the inverted screen test over 120 seconds (Figure 3-5A) to assess the number of rear leg moves performed (Figure 3-5B) and the duration to fall over this period (Figure 3-5C). At 8 months of age, WT mice perform on average almost twice as many rear leg movements as MPS IIIB and also fall later than MPS IIIB mice, but neither effect is significant due to a large variability (Moves; $F_{1,11}$=1.53, p=0.241, Time; $F_{1,11}$=1.05, p=0.327). At 10 months of age the number of rear leg movements

($F_{1,5}$=5.80, p=0.06) and the time to fall from the screen ($F_{1,5}$=5.86, p=0.06) were on the borderline of a significant decrease in MPS IIIB mice. Increased group size would therefore probably have shown a significant effect in both parameters at 10 months.

The horizontal bar test measures motor coordination and grip strength (Figure 3-5D). At 8 months of age there is no significant difference between MPS IIIB and WT mice ($F_{1,11}$=2.34, p=0.15) (Figure 3-5E), but by 10 months of age the MPS IIIB mice are significantly worse at crossing the bar than WT mice ($F_{1,5}$=25.13, p=0.004). This is in agreement with the inverted screen test and suggests that the mice continue to display normal motor skills at 8 months, which are beginning to decline by 10 months of age.

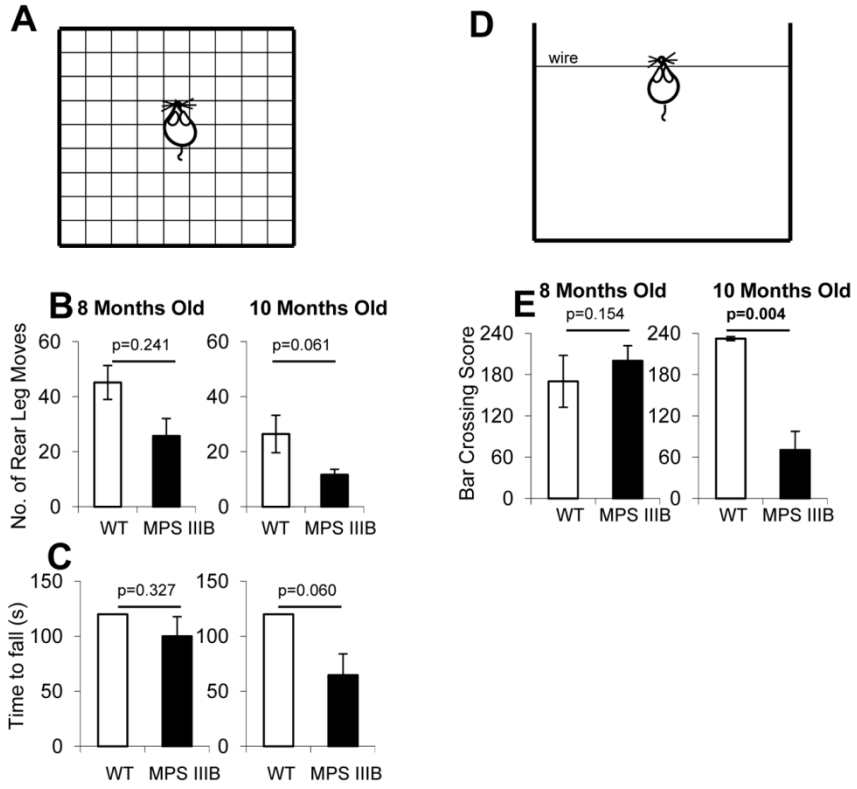

Figure 3-5 Inverted screen and horizontal bar crossing
At 8 months of age 4 WT and 5 MPS IIIB mice and at 10 months of age 3 WT and 4 MPS IIIB mice performed the inverted screed test (A). The number of rear leg moves on the inverted screen (B) and the time spent on the inverted screen (C) was recorded. At 8 months of age 6 WT and 7 MPS IIIB mice and at 10 months of age 3 WT and 4 MPS IIIB mice performed the horizontal bar test (D). The horizontal bar score (E) is a measure of the time of the time taken to cross or fall from the bar. Error bars represent the SEM and *p* values were calculated by t test.

3.5 Discussion

In this work we have been able to determine several behavioural parameters that allow analysis of treatment effects on the neurological deterioration seen in MPS IIIB mice.

The 1 hour open field habituation test performed at the same time of day gives clear genotype separation at 10 minutes and to a greater extent at 1 hour. Eight month old MPS IIIB mice show a significantly increased path length, frequency and duration of rapid exploratory behaviour and reduced frequency and duration of immobility all of which are consistent with a hyperactive phenotype. They also show significantly increased frequency of entry, path length and speed in the central area whilst duration in the centre was unchanged. This suggests that these mice do not have a reduced sense of danger when measured in this test, although the relatively small size of the arena used in this study could have reduced this effect (Crawley 2007). The difference between WT and MPS IIIB mouse behaviour often appeared to diverge from a similar starting point in the first minute or two of the test which led to non-significant differences when observations were measured in the first 3 minutes of the test, as is used by some researchers in the field (Hemsley *et al.* 2005). Although all parameters were significant when measured over the first 10 minutes of the test (with the exception of duration in centre) as is used by many researchers (Cressant *et al.* 2004), 7/8 parameters were more consistently significantly different when measured over the entire 1 hour test period.

Fu *et al.* performed a one hour open field test on 4.5 to 5 month old male and female mice, but did not report activity or anxiety differences except for a decrease in rearing in the second 30 minutes by male mice (Fu *et al.* 2007). It is possible that 4.5 to 5 months of age is too early to detect differences in genotypes but it is also critical that the test is performed at the same time of day and this is not noted in the study. We did not observe differences in any type of rearing activity between 8 month old MPS IIIB and WT mice, however this was only measured over the first 10 minutes. In a 10 minute open field test (Cressant *et al.* 2004), performed at 5 weeks and 7 months of age, no significant genotype differences were observed at 5

weeks of age but significant hyperactivity was seen in MPS IIIB mice at 7 months, in line with the results presented here, which is also consistent with the patient phenotype (Cleary *et al.* 1993).

MPS IIIB mice showed increased frequency of entry, path length and speed in the central area whilst duration in the centre was unchanged. These increases can all be explained by increased hyperactivity causing the mice to move faster and so entering the centre more frequently and travelling further in the same amount of time. MPS IIIB mice have been demonstrated to be less anxious when using the elevated plus maze (Cressant *et al.* 2004), however significant changes were only seen in darkness, and no anxiety differences were seen in a 1 hour open field test in daylight (Fu *et al.* 2007). These discrepancies could be because of the relatively small size of the open field used in our studies (Crawley 2007), or the effect seen in darkness could be an artefact (Cressant *et al.* 2004). To test this hypothesis a larger open field test arena would be required.

In the 24 hour test we observed significant genotype effects in all parameters except duration in the centre, however significance was much less evident compared to the 1 hour test, and increases in immobility frequency and duration in MPS IIIB mice in the 24 hours test are not consistent with the hyperactive behaviour seen in the 1 hour open field test. Differences between MPS IIIB and WT mice appeared to be more evident in daylight than in darkness where few changes were seen. This could be because mice are usually less active in daylight, thus hyperactive behaviour is more easily distinguished during a relatively inactive phase. Interestingly, children with Sanfilippo are reported to have circadian rhythm disturbances, with difficulty in sleeping and frequent night waking and night wandering (Fraser *et al.* 2002; Fraser *et al.* 2005), which would be consistent with the phenotype observed here. In addition, significant periodicity was noted in MPS IIIB mice during the dark phase of the 24 hour test, which may have confounded the ability to distinguish genotypes in darkness. Similar MPS IIIB periodicity, and in particular, a late bout of activity at the transition between light and dark was previously observed by us in a circadian analysis of MPS IIIB mice (Canal *et al.* 2010). MPS IIIB mice show increased frequency and duration of all activity measures with

commensurate reductions in frequency and duration of immobility in the 1 hour open field, which is consistent with sustained hyperactive behaviour. However, in the home cage, although pathlength and frequency of rapid exploratory behaviour is increased, other measures such as duration of rapid activity are not. MPS IIIB mice also have increased frequency and duration of immobility in the home cage. This discrepancy could reflect the fact that there is a nesting box in the home cage where behaviour could not be measured, but this is unlikely as there is no change in the duration of time spent in this area between genotypes. It could instead reflect different types of hyperactive behaviour during exploratory behaviour while the mouse habituates to a novel environment in the 1 hour test compared to normal behaviour in the home cage. The 24 hour home cage behaviour suggests that the mouse is not able to sustain rapid activity over longer periods, and is compensating by spending more time immobile.

The 24 hour data broadly supports the previous circadian analysis by Heldermon *et al* 2007 and ourselves (Heldermon *et al.* 2007; Canal *et al.* 2010; Heldermon *et al.* 2010), but is less effective at separating the phenotypes and less convincingly demonstrates a hyperactive phenotype. Cressant *et al.* also performed a home cage test over a 24 hour period, however that study lacked a 24 hour acclimatisation period, so has an element of habituation (Cressant *et al.* 2004). They observed no difference between MPS IIIB and WT mice in the light but a significant increase in the time the MPS IIIB mice spent in motion in the dark at 4.5 months of age. The open field test could be more consistent than the 24 hour home cage test because the circadian time that testing was performed at (8:30am) is close to a peak of activity that we (Canal *et al.* 2010) have previously observed in MPS IIIB mice just before the lights were switched on in the early morning (7am). This peak can be seen in many of the 24 hour measures in these data as well. In contrast, WT mice are becoming less active as the lights come on. The inverted screen test did not show a significant effect at 8 or 10 months of age but there was a clear trend towards the MPS IIIB mice moving less and being less able to grip the inverted screen. A larger n number for this experiment may make this effect significant. There is a clear significant failure of MPS IIIB mice to complete the horizontal bar crossing test at 10 months of age but no changes at 8 months of age. Taken

together these changes suggest that motor skills are lost late by MPS IIIB mice at 10 months of age, and that it can be variable between mice. A rocking rotarod test was performed by Heldermon *et al*. which also measures motor skills and a significant defect in MPS IIIB mice was seen from 9 months onwards which supports the inverted screen and horizontal bar test results (Heldermon *et al.* 2007; Heldermon *et al.* 2010). An accelerating rotarod test was not reported to show a significant difference between WT and MPS IIIB mice at 5 months of age (Fu *et al.* 2007; Fu *et al.* 2010) or from 1.5 to 13 months of age (Heldermon *et al.* 2007). In our hands motor skills do not deteriorate significantly until 9-10 months of age, thus the horizontal bar test and the inverted screen test only have utility for the long term evaluation of therapies when the mice are at least 9-10 months of age and group sizes are more than 6-7. In addition the observation of urinary retention in these mice from 8-10 months of age onwards (Malinowska *et al.* 2010) is clearly a limiting factor in their ability to perform motor tasks and as such the use of motor tests after 8-9 months of age are more likely to measure this effect than a central dysfunction in motor abilities.

In our previous work on treating MPS IIIB mice with genistein we found similar hyperactivity increases in male MPS IIIB mice, although differences were less marked than females (Malinowska *et al.* 2010). Although this would suggest that female mice may be more consistent for behavioural testing, Fu *et al* 2007 reported vertical activity changes only observed in males (Fu *et al.* 2007). The ability to readily group house females makes them a more economic proposition than males.

3.6 Conclusion

We have demonstrated that it is possible to detect consistently increased hyperactivity and rapid exploratory behaviour in the mouse model of MPS IIIB at 8 months of age using a 1 hour open field test and modest group sizes of 6-7, which is consistent with MPS IIIB patient behaviour. The minimum test time that we would recommend for open field behaviour is 10 minutes, with more consistent data obtained at 60 minutes, and it may be important that tests are carried out close to the time at which lights are switched on in the morning. Home cage behaviour by

contrast is a much less reliable distinguisher of phenotype in MPS IIIB mice and may reflect less sustained hyperactive behaviour. Finally, motor skill evaluations are not likely to yield useful data before 9-10 months of age and can be significantly influenced by gait impairment as a result of urinary retention. This simple protocol will allow the evaluation of potential therapies for MPS IIIB and could prove useful in the development of assessment protocols for other mouse models of neurodegenerative diseases.

Supplementary Video 3-1 MPS IIIB one hour open field

A segment of video recorded during the 1 hour open field test, which shows the median mouse from each group (MPS IIIB on the left, WT on the right) over the median 2 minutes of the test (minutes 29-31). The clip is shown at 4 times normal speed. Please see enclosed CD.

Chapter 4 - MPS IIIA Behaviour

Female Mucopolysaccharidosis IIIA Mice Exhibit Hyperactivity and a Reduced Sense of Danger in the Open Field Test

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The definitive version can be found at

http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0025717

and the published version can be found in the appendix.

4.1 Abstract

Reliable behavioural tests in animal models of neurodegenerative diseases allow us to study the natural history of disease and evaluate the efficacy of novel therapies. Mucopolysaccharidosis IIIA (MPS IIIA or Sanfilippo A), is a severe, neurodegenerative lysosomal storage disorder caused by a deficiency in the heparan sulphate catabolising enzyme, sulphamidase. Undegraded heparan sulphate accumulates, resulting in lysosomal enlargement and cellular dysfunction. Patients suffer a progressive loss of motor and cognitive function with severe behavioural manifestations and premature death. There is currently no treatment. A spontaneously occurring mouse model of the disease has been described, that has approximately 3% of normal enzyme activity. Behavioural phenotyping of the MPS IIIA mouse has been previously reported, but the results are conflicting and variable, even after full backcrossing to the C57BL/6 background. Therefore we have independently backcrossed the MPS IIIA model onto the C57BL/6J background and evaluated the behaviour of male and female MPS IIIA mice at 4, 6 and 8 months of age using the open field test, elevated plus maze, inverted screen and horizontal bar crossing at the same circadian time point. Using a 60 minute open field, we have demonstrated that female MPS IIIA mice are hyperactive, have a longer path length, display rapid exploratory behaviour and spend less time immobile than WT mice. Female MPS IIIA mice also display a reduced sense of danger and spend more time in the centre of the open field. There were no significant differences found between male WT and MPS IIIA mice and no differences in neuromuscular strength were seen with either sex. The altered natural history of behaviour that we observe in the MPS IIIA mouse will allow more accurate evaluation of novel therapeutics for MPS IIIA and potentially other neurodegenerative disorders.

4.2 Introduction

Mucopolysaccharidosis IIIA (MPS IIIA, OMIM #252900), or Sanfilippo Type A, is an autosomal recessive lysosomal storage disorder that affects 0.82 in 100,000 live births in the United Kingdom (Heron *et al.* 2011). The disease is characterised by severe and progressive loss of cognitive and motor functions, behavioural difficulties and eventually death in the second decade of life, although the severity and progression of the disease varies widely (Meyer *et al.* 2007; Valstar *et al.* 2010; Heron *et al.* 2011). MPS IIIA is caused by mutations in the *SGSH* gene that result in deficiency of the N-sulphoglucosamine sulphohydrolase enzyme (sulphamidase, EC 3.10.1.1) and subsequent accumulation of undegraded heparan sulphate, lysosomal enlargement and cellular and organ dysfunction (Kresse *et al.* 1972; Kresse 1973; Scott *et al.* 1995; Valstar *et al.* 2008). Patients exhibit progressive neurodegeneration and behavioural problems including hyperactivity, a reduced sense of danger, aggression and sleep disturbances (Cleary *et al.* 1993; Bax *et al.* 1995; Fraser *et al.* 2002; Fraser *et al.* 2005; Valstar *et al.* 2008; Malm *et al.* 2010).

Although there are no current therapies, several strategies are in development for MPS IIIA or the phenotypically indistinguishable MPS IIIB disease, including substrate reduction therapy (Roberts *et al.* 2007; Malinowska *et al.* 2009; Malinowska *et al.* 2010), intrathecal enzyme replacement therapy (Gliddon *et al.* 2004; Savas *et al.* 2004; Hemsley *et al.* 2007; Hemsley *et al.* 2008b; Hemsley *et al.* 2009b) and gene therapy with lentiviral (McIntyre *et al.* 2010), adenoviral (Lau *et al.* 2010b) or adeno-associated-viral (Fraldi *et al.* 2007) vectors. Most strategies make use of the ability of exogenous enzyme to complement affected cells, however the presence of the blood brain barrier limits efficient enzyme distribution. Biomarkers for MPS IIIA or related diseases are still in development (Langford-Smith *et al.* 2010; Langford-Smith *et al.* 2011c), thus the accurate evaluation of neurodegeneration using behavioural phenotyping in the mouse model of disease is paramount. A spontaneously occurring mouse model of MPS IIIA on a mixed 129SvJ, C57BL/6, SJL, and CD1 background has been previously described with around 3% of normal enzyme activity and exhibiting many of the features observed in patients (Bhaumik *et al.* 1999). The MPS IIIA mice exhibit severe neuropathology characterised by enlarged lysosomes, primary storage of HS, secondary storage of GM2 and GM3

gangliosides and cholesterol and chronic neuroinflammation (Bhaumik *et al.* 1999; McGlynn *et al.* 2004; Savas *et al.* 2004; Fraldi *et al.* 2007; Arfi *et al.* 2011). The MPS IIIA mice are euthanized between 9 and 12 months of age due to urine retention, a phenotype not seen in the patients but also seen in MPS IIIB mice (Gografe *et al.* 2009). The MPS IIIA mouse model has been backcrossed to the C57BL/6 background by Professor John Hopwood's Lysosomal Diseases Research Unit (Crawley *et al.* 2006) and to the C57BL/6J background by Jackson laboratories (Website)(Website)(Website)(Website)(Website)(Jackson Laboratories website).

Although the behaviour of the MPS IIIA mouse model has been evaluated in the open field test at several ages, using different sexes, by several groups and on different strain backgrounds, there is a remarkable amount of variability in the outcome of the test as outlined in Table 4-1.

Using the mixed background mouse, at many of the time points measured, no differences are observed between either male or female WT and MPS IIIA mice, with initial findings of hyperactivity and later hypoactivity in males (Hemsley *et al.* 2005; Hemsley *et al.* 2007). After back crossing the MPS IIIA mice on to a C57BL/6 background, the behaviour was extensively studied by Crawley *et al.* at different time points. Hyperactivity in males was observed at 12 weeks of age, and females at 22 and 32 weeks of age, although these mice were not näively tested (Crawley *et al.* 2006). At most time points, no differences were seen. However male, but not female, MPS IIIA mice were shown to be hypoactive at some time points in the work of Lau *et al* (Lau *et al.* 2008) and male MPS IIIA mice were hypoactive at 15 weeks of age in a more recent paper by Lau *et al* (Lau *et al.* 2010a). Hypoactivity in male mice was also observed at some time points in McIntyre *et al.* (McIntyre *et al.* 2010). In columns 7, 8, 9 and 10 of Table 4-1 (marked as bold) we have also presented markedly variable outcomes of observations of locomotor behaviour of MPS IIIA and WT mice that received control intracranial injections (Fraldi *et al.* 2007; Hemsley *et al.* 2007; Hemsley *et al.* 2009a; McIntyre *et al.* 2010).

Patients with MPS IIIA are believed to have a reduced sense of danger, which can be inferred in mice by the amount of time spent in the centre of the open field test or by use of the elevated plus maze test, both of which can be used to measure

their tendency to avoid open spaces and remain close to cover (thigmotaxis). No difference in the time in centre of the open field was detected by Lau *et al.* (Lau *et al.* 2008), however this could be due to the small size of the open field used. In the elevated plus maze, male MPS IIIA mice were reported to display reduced anxiety with a greater proportion of their path length (distance travelled) spent in the open arms at some time points but not others (Lau *et al.* 2008; Lau *et al.* 2010a).

Overall, there are discrepancies in behaviour of this mouse model that could be due to gender differences, the age of testing and the methodology used to perform the tests. Therefore we have attempted to perform a standardised analysis of MPS IIIA mice by first backcrossing them to the C57BL/6J background for over 10 generations and subsequently testing a cohort of MPS IIIA and WT mice at the same circadian time point at 4, 6 and 8 months (16, 24 and 32 weeks) of age in the 60 minute open field test, the elevated plus maze, as well as several other neuromuscular evaluations that we have previously shown to be effective for phenotyping MPS IIIB mice (Langford-Smith *et al.* 2011b). The female MPS IIIA mice were hyperactivie, had a reducued sense of danger and no neuromuscular differences. The open field test performed at the same point in the circadian rhythm was a consistent, sensitive and reliable behavioural test for the evaluation of novel therapeutic strategies in MPS IIIA mice.

Table 4-1 Summary of the significant differences in the open field activity of MPS IIIA mice in the literature

Paper	Hemsley *et al.*	Hemsley *et al.*	Lau *et al.*	Lau *et al.*	McIntyre *et al.*	Crawley *et al.*	**** Hemsley et al.***	**** Hemsley et al.***	**** McIntyre et al.***	**** Fraldi et al.***	This Study	
Year	2005	2007	2008	2010	2010	2006	***2007***	***2009***	***2010***	***2007***		
Background	Mixed	Mixed	C57BL/6	C57BL/6	C57BL/6	C57BL/6	***C57BL/6***	***C57BL/6***	***C57BL/6***	***C57BL/6***	C57BL/6J	
Measure	Line Crossing	Line Crossing	Zone Entries	Path Length	Line crossing	Line Crossing	***Line Crossing***	***Path Length***	***Line crossing***	***Path Length***	Path Length	
Sex	Male Female	Male	Male Female	Male	Male	Male Female	***Male***	***Male***	***Male***	***Male***	Male	Female
3	Hyper Hyper		NS NS									
4						NS Hypo						
5			NS NS									
6	Hypo NS	Hypo				NS NS						
8						NS NS	***NS* +Hypo****					
10	NS NS		Hypo NS			NS NS				***NS****		
12						Hyper NS	***NS* +Hypo****	***Hypo****				
15	Hypo NS		NS NS	Hypo		NS NS				***NS****		
16											NS	Hyper
18			Hypo NS			NS NS	***Hypo****			***NS****		
20	NS NS				Hypo				***NS****			
21										***Hyper****		
22			Hypo NS			NS Hyper						
24					NS				***NS****		NS	Hyper
25						NS NS						
28					NS				***NS****			
32						NS Hyper					NS	Some Hyper
40	NS NS					NS NS						

Hyper indicates significant hyperactive behaviour and hypo indicates significant hypoactive behaviour in the open field test. NS indicates no significant difference and * indicates mice that have recieved intracranial injections. Control injections contained: 2.5µl of 50mM sodium acetate, 100mM sodium chloride at pH 5.0 in Hemsley et al 2007, 4µl 10mM sodium phosphate, 140mM sodium chloride pH 7.0 in Hemsley et al. 2009, 5µl 0.9% (w/v) sodium chloride in McIntyre et al. 2010 and 1µl GFP adeno-associated viral vector in Fraldi et al. 2007. The work of Hemsley et al. 2007 contained multiple control treated groups and where there were differences between groups, both have been shown. Where female mice are not mentioned or cells are blank no testing was performed.

4.3 Methods

4.3.1 Mouse Maintenance

The MPS IIIA mouse colony was maintained through heterozygous breeding at the University of Manchester, all procedures were ethically approved by the University of Manchester Ethical Review Process Committee and in accordance with the UK Home Office regulations under project licence PPL 40/3117. Mice were housed in individually ventilated cages, had access *ad libitum* to food and water and were in a 12 hour light and dark cycle. Male mice were singly housed at 14 weeks of age due to aggressive tendencies but female mice remained housed in groups of 4-6. The MPS IIIA mice have been backcrossed from the original mixed 129SvJ, C57BL/6, SJL, and CD1 background (Bhaumik *et al.* 1999) onto the C57BL/6J background by more than 10 generations of backcrossing with C57BL/6J mice (Harlan, UK) and were maintained by heterozygous breeding. WT and MPS IIIA littermates have been used in all behavioural experiments.

4.3.2 Genotyping MPS IIIA mice

MPS IIIA mice have a G to A mutation in the SGSH gene which removes a *Msp*A1I restriction enzyme digestion site. Genotyping is performed by PCR amplification of DNA followed by *Msp*A1I digestion and observation of the size of DNA fragments produced. Genomic DNA was extracted from ear punches using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma Aldrich) following the manufacturer's instructions and SGSH was amplified by PCR using the forward primer 5' GTGTTCCCTGCCTGCTCAC 3' and reverse primer 5' CCAGTCCCCTCATCCCACTA 3'. The DNA was digested with *Msp*A1I (New England Biolabs, UK) and the DNA fragments were separated by 2% agarose gel electrophoresis. The genotype was determined from the pattern of DNA fragments; wild type (WT) reveals 199bp, 118bp and 78bp fragments, mutant (MUT) 317bp and 78 bp fragments and heterozygous (HET) 317bp, 199bp, 118bp and 78bp.

4.3.3 Behavioural Testing

At 4, 6 and 8 months (16, 24 and 32 weeks) of age, the same cohorts of 10 male WT and 10 male MPS IIIA mice and 10 female WT and female 11 MPS IIIA mice were

analysed with the following behavioural tests. 1.5 hours into the 12 hour light phase the mice were dropped into the centre of an open field arena (width 450mm, depth 450mm, height 500mm) made of matt white acrylic (Figure 4-1A). The behaviour was recorded for 60 minutes using a digital camcorder (Sony) and analysed using Top Scan software (Clever Sys. Inc., USA). The path length, frequency (number of times) and duration of rapid exploratory behaviour (speed >100mm/s), frequency and duration of immobility (speed <5mm/s) and frequency of entering the centre and duration in the centre (75mm from each edge) was analysed. Rearing in the open field was analysed by counting the number of unsupported rears (front paws off the floor) and supported rears (front paws on the wall) in the first 10 minutes. The same researcher performed all experiments and was blinded to genotype.

After a 30 minute rest, the mice were placed onto the end of an open arm of an elevated plus maze. The maze was constructed of matt white acrylic, comprised of four 500mm long by 100mm wide arms, two of which were enclosed by 500mm high walls, and was raised on a stand 500mm off the floor (Figure 4-5A). Mice were placed so they faced towards the centre of the maze and were given 10 minutes to explore the maze before returning to a cage. The maze was cleaned using 70% ethanol. 30 minutes later the mice were tested for a second time, in the same manner. The results were analysed using Top Scan software (Clever Sys) to examine the amount of time spent on the open arm, the percentage of path length in the open arm, and the percentage of open arm entries.

30 minutes later the inverted screen test was performed, as described previously (Figure 4-6A) (Langford-Smith *et al.* 2011b). In brief, at 4, 6 and 8 months of age the mouse was placed on a 470mm square with a 13mm square wire mesh. The screen was then rotated through 180° over 1-2 seconds. The mouse was then suspended upside down over a padded surface; the rear leg moves were counted and the duration suspended was recorded up to 2 minutes.

One hour later the horizontal bar test was performed as described previously (Figure 4-6B) (Malinowska *et al.* 2010; Langford-Smith *et al.* 2011b). In brief, a 2mm diameter, 300mm long metal wire was secured between 2 posts, 320mm above a

padded surface. The mouse was allowed to grip the centre of the wire and the time taken to fall or to reach the side was recorded up to 2 minutes. The test was repeated three times as a training run followed by a 10 minute rest before three test runs. The results were scored as follows: crossing the bar in x seconds was scored as 240-x, remaining on the bar was scored as 120 and falling off the bar after y seconds was recorded as y.

4.3.4 Urine Retention

Upon sacrifice 9 male WT, 8 male MPS IIIA, 7 female WT and 8 female MPS IIIA mice at 8 months (32 weeks) of age were dissected and the urine was removed from the bladder and volume measured using an insulin syringe (BD).

4.3.5 Statistical Analysis

Statistical analysis was performed using JMP software (SAS Institute Inc, Cary, NC, USA) and analysed by MANOVA with repeated measures for overall significances and by one or two way ANOVA, as appropriate, with Tukey post hoc tests at individual time points. Significance was set at $p \leq 0.05$. For the MANOVA analysis the Genotype significance determines if there is a difference between WT and MPS IIIA mice irrespective of the age of the mice, the Time significance determines if there is a change in the behaviour of the mice at different ages irrespective of the genotype and the Genotype*Time significance determines if MPS IIIA mouse behaviour changes over time in a different manner to WT mice.

4.4 Results

Cohorts of male and female, WT and MPS IIIA mice (n=10-11 per group) were monitored in a 60 minute open field test as outlined in Figure 4-1A. The path length minute by minute over 60 minutes is shown at 4 months (16 weeks) in Figure 4-1B, 6 months (24 weeks) in Figure 1C and at 8 months (32 weeks) in Figure 1D. The behaviour in the first 3 minutes, 10 minutes and 60 minutes is presented to allow comparison against published data and to identify the minimum amount of time required for statistically significant analyses. At 4 months of age, female MPS IIIA mice were significantly hyperactive and travel almost twice as far as the WT

mice over 60 minutes (p=0.037; Figure 4-1B). Examining the behaviour over the first 3 minutes showed no difference and no trend in female behaviour, but over 10 minutes there was a non-significant trend towards hyperactivity. No difference was found in the path length of male WT or MPS IIIA mice at 4 months.

At 6 months of age the same female MPS IIIA mice demonstrated increased path length with significant differences after 10 minutes (p=0.030) and greater differences after 60 minutes (p=0.005; Figure 4-1C). No difference was detected between male WT and MPS IIIA mice (Figure 4-1C).

At 8 months of age there was a trend for the female mice to be hyperactive but no significant differences were found after 3 (p=0.73), 10 (p=0.15) or 60 minutes (p=0.18; Figure 4-1D). Similarly, no significant differences were found with male mice after 3, 10 or 60 minutes. Overall, using MANOVA repeated measure analysis, a significant genotype difference (p=0.011), time difference ($p=1.2x10^{-7}$) and a time*genotype difference (p=0.021) were found. This indicates that overall, MPS IIIA mice are significantly more hyperactive than WT mice, that this changes with time and that MPS IIIA and WT mice change their behaviour in a different manner over time.

The number of unsupported rears (front paws off the floor), supported rears (front paws on a wall) and total rears were counted manually in the first 10 minutes of the open field test. The only significant change observed was a decrease in the number of unsupported rears by female MPS IIIA mice at 6 months of age (Figure 4-1F). However, given that there were no other significant genotype effects, this may be just a chance occurrence (Figure 4-1E and G). MANOVA repeated measure analysis of the data showed no significant genotype effect in the three rearing measures, but there was a significant decline in all measures with time (unsupported; p=0.004, supported and total p=0.00003) irrespective of genotype.

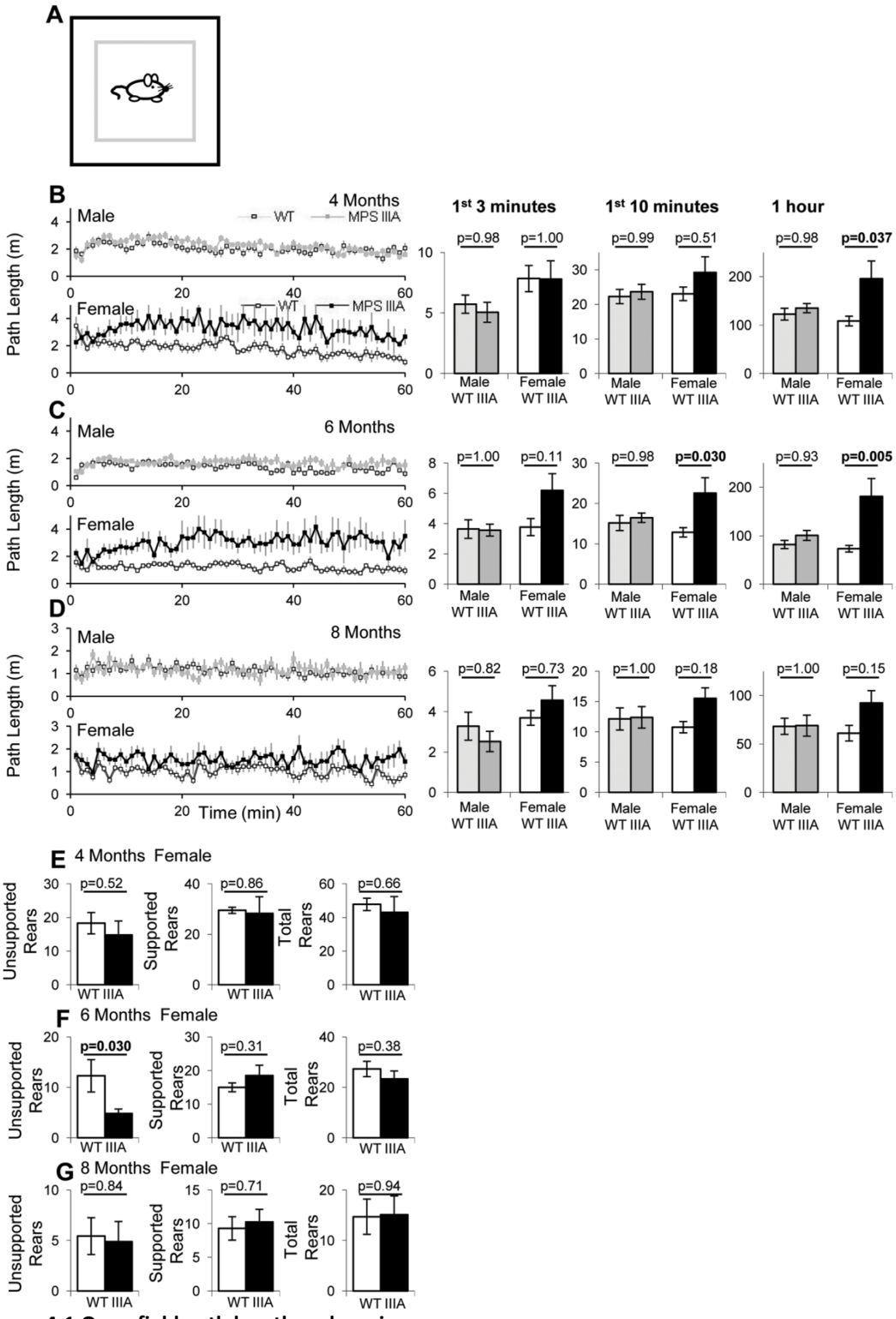

Figure 4-1 Open field path length and rearing

At 4, 6 and 8 months (16, 24 and 32 weeks) of age, 10 WT male (light grey squares), 10 MPS IIIA male (dark grey squares), 10 WT female (white squares) and 11 MPS IIIA female (black squares) mice were placed in the open field and the behaviour was recorded for 60 minutes (A). The results of the open field behaviour are presented as a 60 minute period with the average of every minute presented and as a bar chart of the first 3 minutes, first 10 minutes and the whole hour. Error bars represent the standard error of the mean (SEM). p values were calculated by 2 Way ANOVA. The mean average path length in metres at 4 (B), 6 (C) and 8 (D) months have been presented. The number of unsupported, supported and total rears in the first 10 minutes at 4 (E), 6 (F) and 8 (G) months of age have also been presented.

Rapid exploratory motion was analysed by measuring the frequency and duration of speed over 100mm/s (Figure 4-2A-F). At 4 months of age, female MPS IIIA mice show significant increases in both the frequency (p=0.048; Figure 4-2A) and duration (p=0.05; Figure 4-2D) of rapid exploration over 60 minutes. However, no significant differences were observed after 3 or 10 minutes, although a trend was detected after 10 minutes (Figure 4-2A). No significant differences were found between male MPS IIIA or WT mice at 4 months of age.

At 6 months of age, there was a trend towards increased frequency (Figure 4-2B) and duration (Figure 4-2E) of rapid exploratory motion after 3 minutes in the female MPS IIIA mice (p=0.23, p=0.10). After 10 (p=0.021, p=0.034) and 60 minutes (p=0.0003, p=0.0057), female MPS IIIA mice had significantly increased frequency (Figure 4-2B) and duration (Figure 4-2E) of rapid exploratory motion over 100mm/s. No significant differences were observed between male WT and MPS IIIA mice. This rapid exploratory behaviour is visible in supplementary video 4-1 which shows the median female WT and MPS IIIA mouse at 6 months of age at 4 times normal speed.

At 8 months of age, female MPS IIIA mice show a trend towards increased frequency of rapid exploratory motion over 60 minutes (p=0.18) (Figure 4-2C), with a trend to increase in duration after 10 minutes (p=0.083) and a significant increase after 60 minutes (p=0.02) (Figure 4-2F). Male MPS IIIA and WT mice were indistinguishable. Using MANOVA repeated measure analysis, there was a significant difference between WT and MPS IIIA mice over all three time points for frequency (Genotype; p=0.004) and duration (Genotype; p=0.013) of rapid exploratory motion. There was a significant decline in frequency (Time; $p=9.7x10^{-15}$) and duration (Time, $p=4.5x10^{-8}$) of rapid exploratory motion with time and also between genotypes over time for frequency (Time*Genotype p=0.00005) and duration (Time*Genotype, p=0.036).

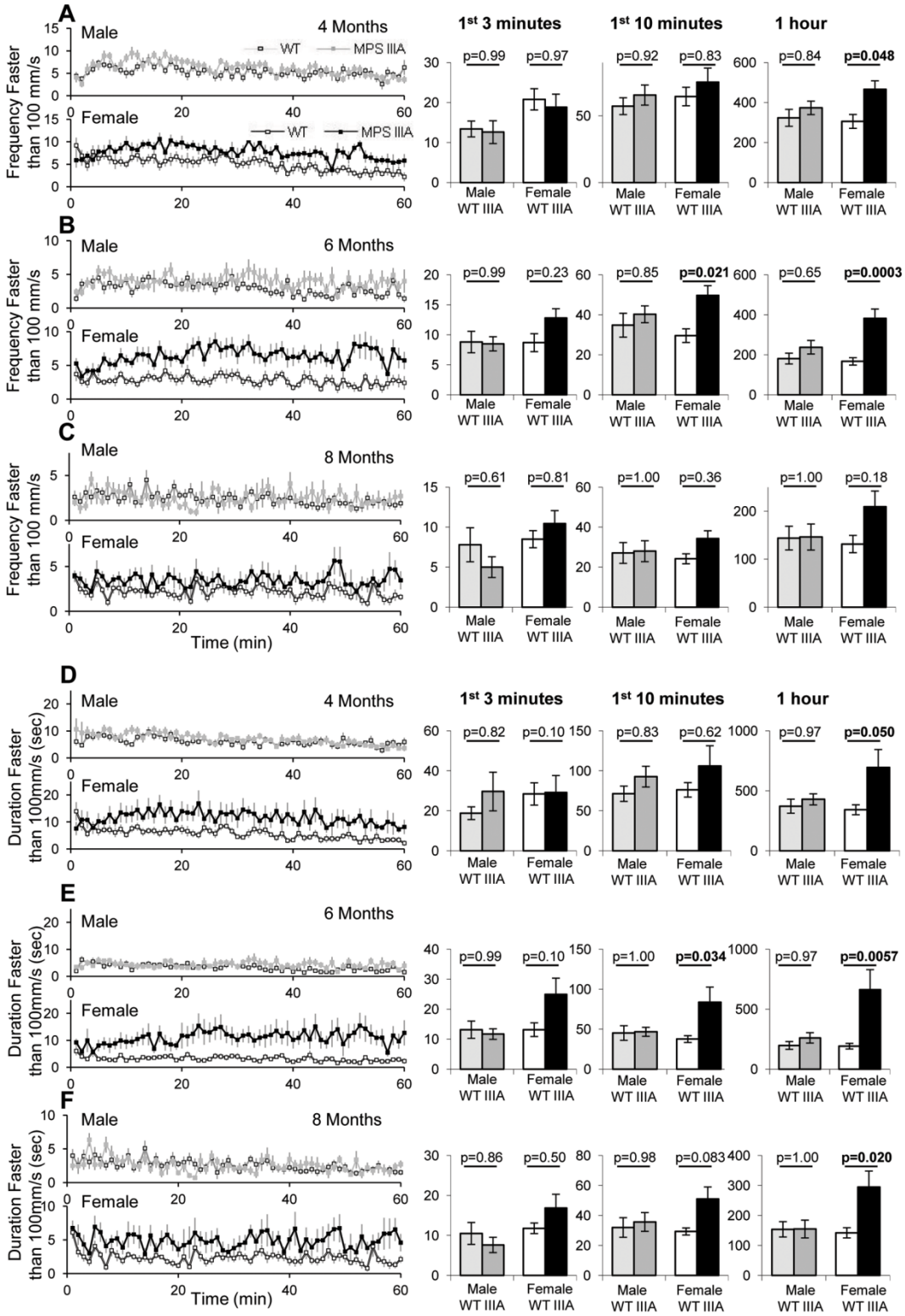

Figure 4-2 Open field rapid exploratory behaviour

At 4, 6 and 8 months of age 10 WT male (light grey squares), 10 MPS IIIA male (dark grey squares), 10 WT female (white squares) and 11 MPS IIIA female (black squares) mice were placed in the open field and the behaviour was recorded for 60 minutes. The results of this test are presented as a 60 minute period with the average of every minute presented and as a bar chart of the first 3 minutes, first 10 minutes and the whole hour. Error bars represent the SEM. p values were calculated by 2 Way ANOVA. The frequency of moving faster than 100mm/s at 4 (A), 6 (B) and 8 (C) months and the duration spent moving faster than 100mm/s at 4 (D), 6 (E) and 8 (F) months of age have been presented.

The frequency and duration the mice spent immobile was also recorded (Figure 4-3A-F). There were no significant differences in frequency of immobility between either male or female MPS IIIA and WT mice at any time point. However, female WT mice had significantly increased duration of immobility over 60 minutes at 4 (p=0.005) and 6 months (p=0.004) but not at 8 months (p=0.70). Male MPS IIIA or WT mice had indistinguishable duration of immobility at all time points measured. Using MANOVA repeated measures there was an overall increase in the frequency of WT immobility over MPS IIIA mice over the three time points (Genotype; p=0.027), but no change with time (Time; p=0.543), or genotype with time interaction (Genotype*Time; p=0.636). MANOVA repeated measures analysis of duration of immobility demonstrated a significant genotype effect over the three time points (Genotype; p=0.05), that changes significantly with time (Time; $p=1.5x10^{-9}$) and changes differently with time between the two genotypes (Genotype*Time; p=0.030).

Mice being a prey species tend to display thigmotaxis, remaining close to the sides of an open field arena. Increased frequency and duration of time spent in the centre of the open field test therefore demonstrates reduced thigmotaxis and this is commonly considered to be a measure of reduced anxiety or reduced sense of danger that the animal experiences (Simon *et al.* 1994). Male MPS IIIA and WT mice showed no significant differences in frequency of centre entries at any age (Figure 4-4A, B and C). At 4 months of age, female MPS IIIA mice showed significantly increased centre entries at 60 minutes (p=0.0045). At 6 months female MPS IIIA mice entered the centre significantly more at 3, 10 and 60 minutes (p=0.057, p=0.023, p=0.0005; Figure 4-4B). At 8 months of age female MPS IIIA showed no significant increases in centre entries (p=0.37) (Figure 4-4C). Using MANOVA MPS IIIA mice showed significantly increased centre entries (Genotype; p=0.003), which changed significantly over time (Time; $p=2.3x10^{-11}$) and between genotypes over time (Genotype*Time; p=0.0013).

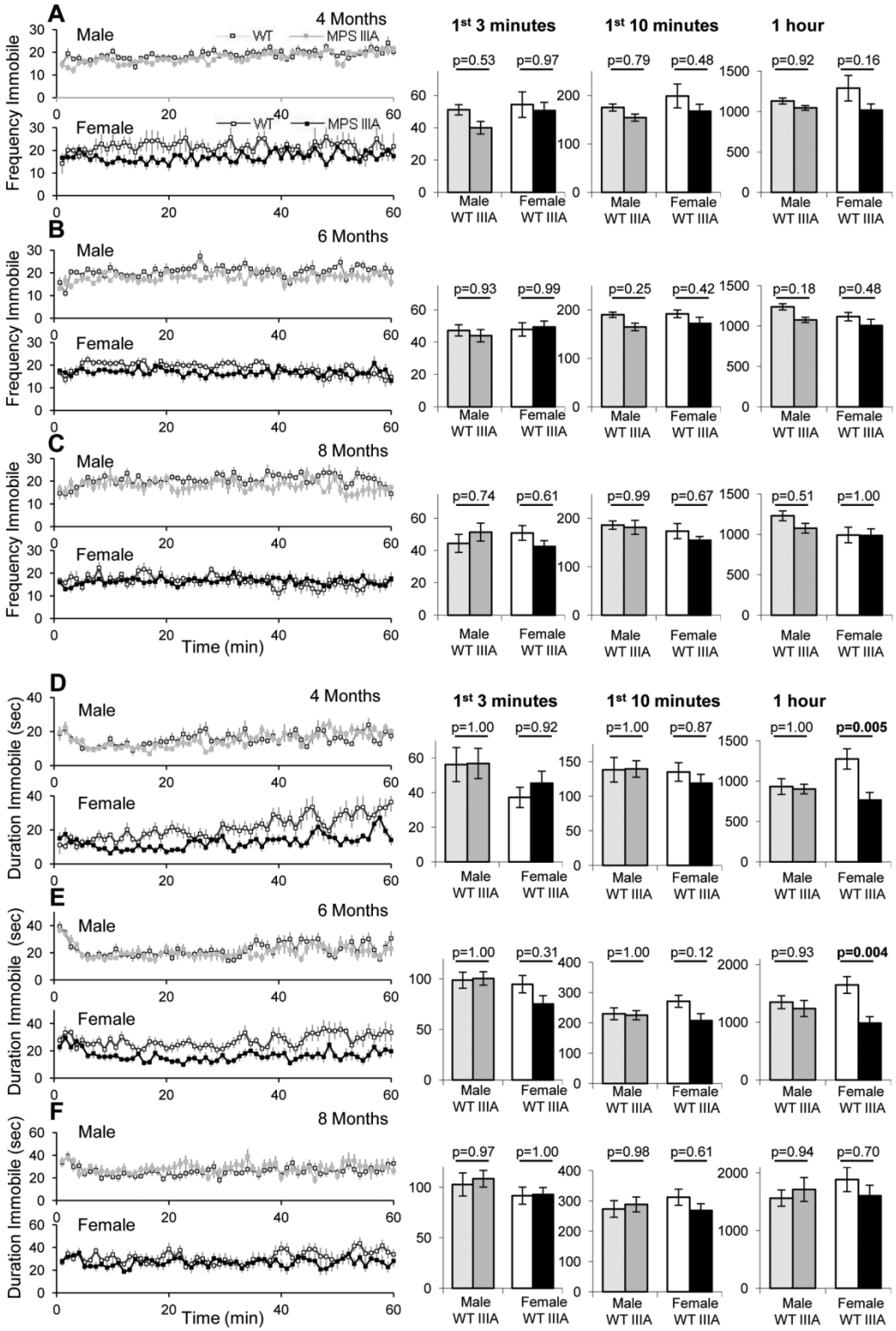

Figure 4-3 Open field immobile behaviour

At 4, 6 and 8 months of age 10 WT male (light grey squares), 10 MPS IIIA male (dark grey squares), 10 WT female (white squares) and 11 MPS IIIA female (black squares) mice were placed in the open field and the behaviour was recorded for 60 minutes. The results of this test are presented as a 60 minute period with the average of every minute presented and as a bar chart of the first 3 minutes, first 10 minutes and the whole hour. Error bars represent the SEM. p values were calculated by 2 Way ANOVA. The frequency of not moving at 4 (A), 6 (B) and 8 (C) months and the duration spent not moving at 4 (D), 6 (E) and 8 (F) months of age have been presented.

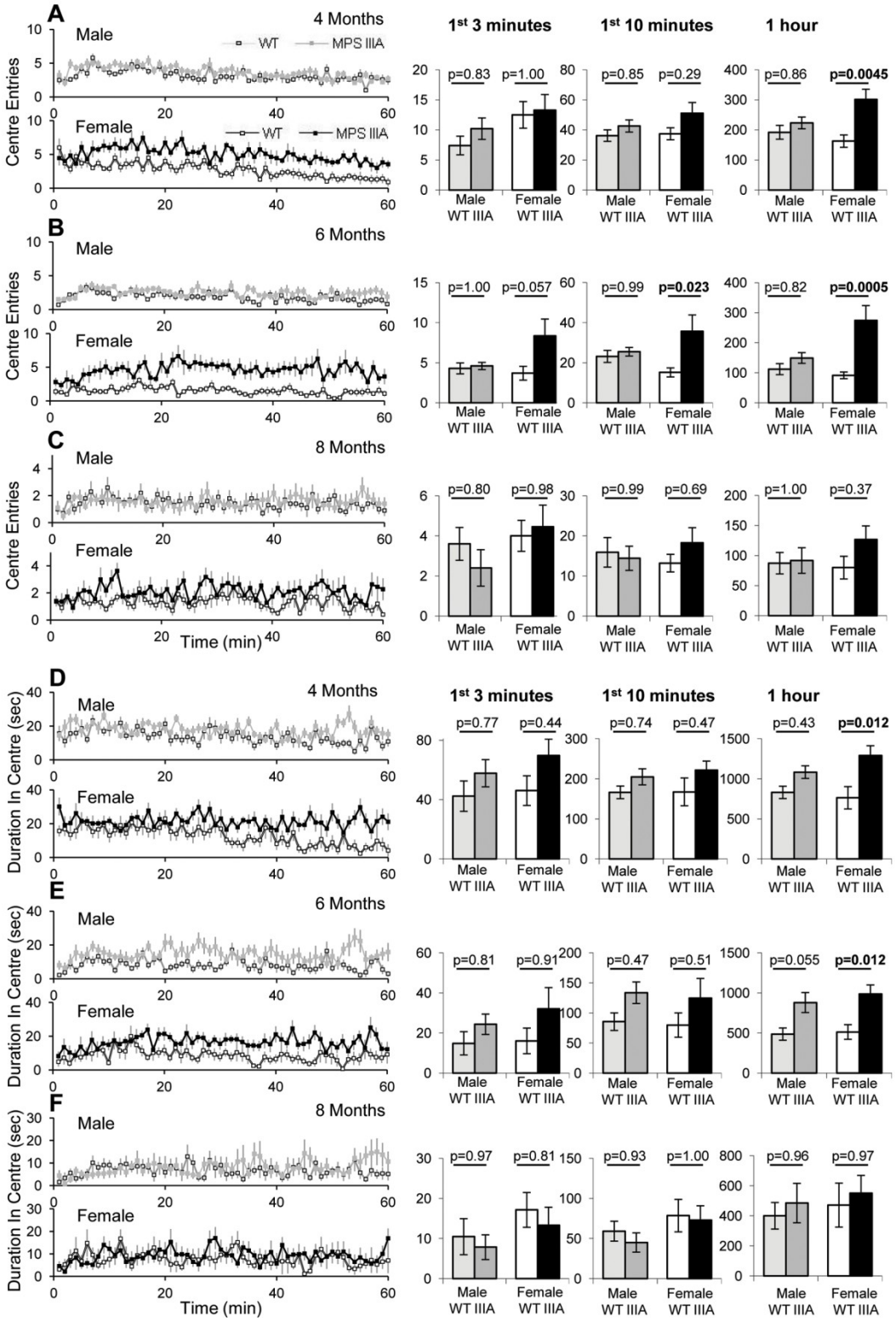

Figure 4-4 Open field sense of danger behaviour

At 4, 6 and 8 months of age 10 WT male (light grey squares), 10 MPS IIIA male (dark grey squares), 10 WT female (white squares) and 11 MPS IIIA female (black squares) mice were placed in the open field and the behaviour was recorded for 60 minutes. The results of this test are presented as a 60 minute period with the average of every minute presented and as a bar chart of the first 3 minutes, first 10 minutes and the whole hour. Error bars represent the SEM. p values were calculated by 2 Way ANOVA. The frequency of entering the centre at 4 (A), 6 (B) and 8 (C) months and the duration spent in the centre at 4 (D), 6 (E) and 8 (F) months of age have been presented.

The duration spent in the centre has also been analysed. At 4 months of age, female MPS IIIA mice spent significantly more time in the centre than WT, after 60 minutes (p=0.012; Figure 4-4D). Males were not significantly different. At 6 months of age female MPS IIIA mice had increase duration in the centre after 60 minutes (p=0.012) and showed an almost significant trend in male mice (p=0.055; Figure 4-4E). At 8 months of age there are no significant differences between genotypes (Figure 4-4F). By MANOVA repeated measure analysis MPS IIIA mice spend significantly increased duration in the centre area (Genotype; p=0.001), this changes significantly over time (Time; $p=2.5x10^{-6}$) and between genotypes over time (Time*Genotype; p=0.005).

Thirty minutes after behavioural analysis in the open field, the same cohort of mice were tested on an elevated plus maze (Figure 4-5A) for ten minutes, followed by a 30 minute rest and another 10 minute trial. Several parameters were measured (frequency of entering open arm, percentage of entries into the open arm, path length in open arm, percentage of path length in open arm and time in open arm) but none reached significance. Here we have presented the percentage of entries to the open arms, percentage of path length in the open arms and duration in the open arms as measure of anxiety (Figure 4-5B, C, D), which is representative of many of the other measures. At 4, 6 and 8 months of age there was no significant difference in male or female WT or MPS IIIA open arm entries (Figure 4-5B, C, D). At 4, 6 and 8 months there was no difference between WT and MPS IIIA mice in the repeat elevated plus maze and no difference between first and second elevated plus maze tests except that in the second test, all the mice performed fewer entries and had a shorter path length (Figure 4-6A-C). Statistical analysis by MANOVA showed no significant difference in genotype, or time but there was a significant difference in how the genotypes behaved over time in the percentage of path length in the open arm (p=0.019) as the MPS IIIA mouse path length decreased at 8 months but the WT increased.

The inverted screen test (Figure 4-7A) and horizontal bar crossing test (Figure 4-7B) were also performed. The inverted screen test measures neuromuscular strength and the bar crossing test measures both neuromuscular strength and motor

coordination. The bar crossing test showed no significant differences between WT and MPS IIIA of either sex at 4, 6 or 8 months of age (Figure 4-7C-E). Over time, there was a significant decrease in the number of moves and time spent on the inverted screen ($p=1.7x10^{-6}$, $p=1.2x10^{-5}$), and a significant sex difference in the number of moves with females moving more (p=0.004). Bar crossing showed no genotype effect and no time effect but there was a significant Time*Sex effect (p=0.044), as the female score decreased over time but the male score increased at 6 months and then decreased at 8 months.

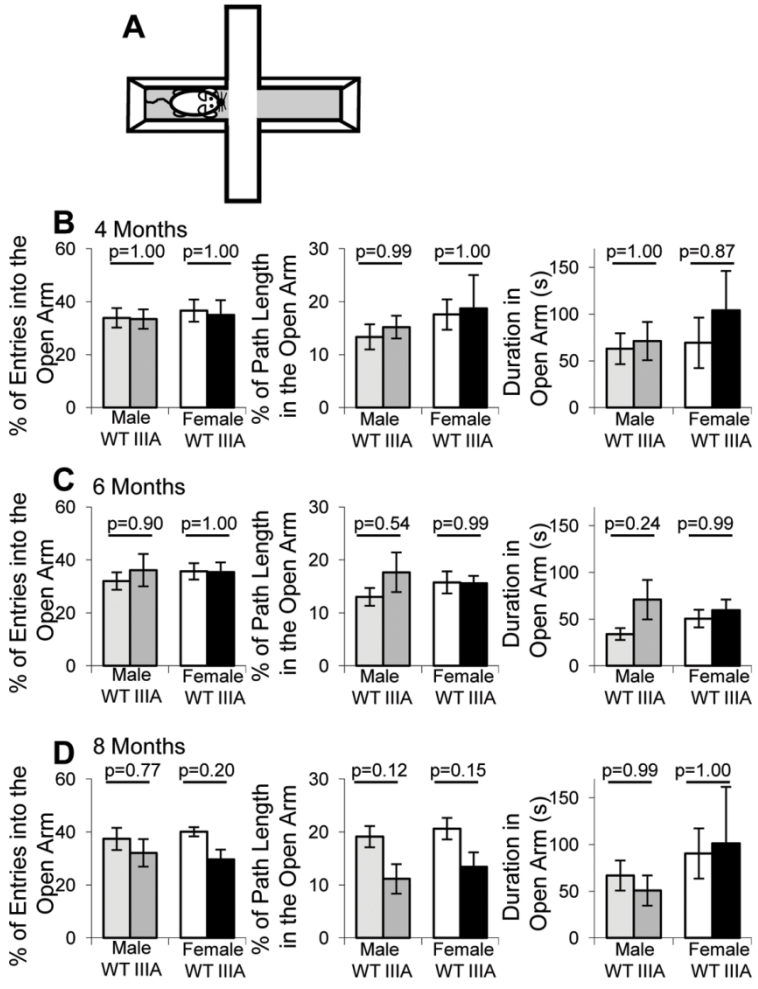

Figure 4-5 Elevated plus maze behaviour

At 4, 6 and 8 months of age 7-11 WT male (light grey bars), MPS IIIA male (dark grey bars), WT female (white bars) and MPS IIIA female (black bars) mice were placed on the elevated plus maze (A) and the behaviour was recorded for 10 minutes. After a 30 minute rest, the test was repeated. The results of the first test are presented as the mean of each measure with error bars representing the SEM. p values were calculated by 2 Way ANOVA. The following measures have been presented; the percentage of total entries that were into the open arm, the percentage of the path length in the open arm and the duration spent on the open arm at 4 (B), 6 (C) and 8 (D) months of age.

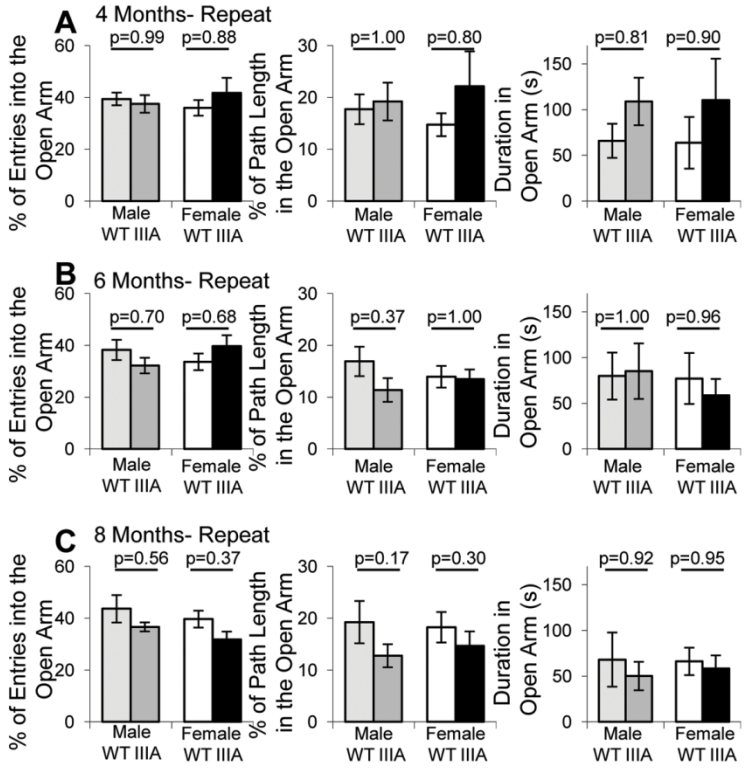

Figure 4-6 Repeat elevated plus maze behaviour

At 4, 6 and 8 months of age 7-11 WT male (light grey bars), MPS IIIA male (dark grey bars), WT female (white bars) and MPS IIIA female (black bars) mice were placed on the elevated plus maze (A) and the behaviour was recorded for 10 minutes. After a 30 minute rest, the test was repeated. The results of the repeat test are presented as the mean of each measure with error bars representing the SEM. p values were calculated by 2 Way ANOVA. The following measures have been presented; the percentage of total entries that were into the open arm, the percentage of the path length in the open arm and the duration spent on the open arm at 4 (A), 6 (B) and 8 (C) months of age.

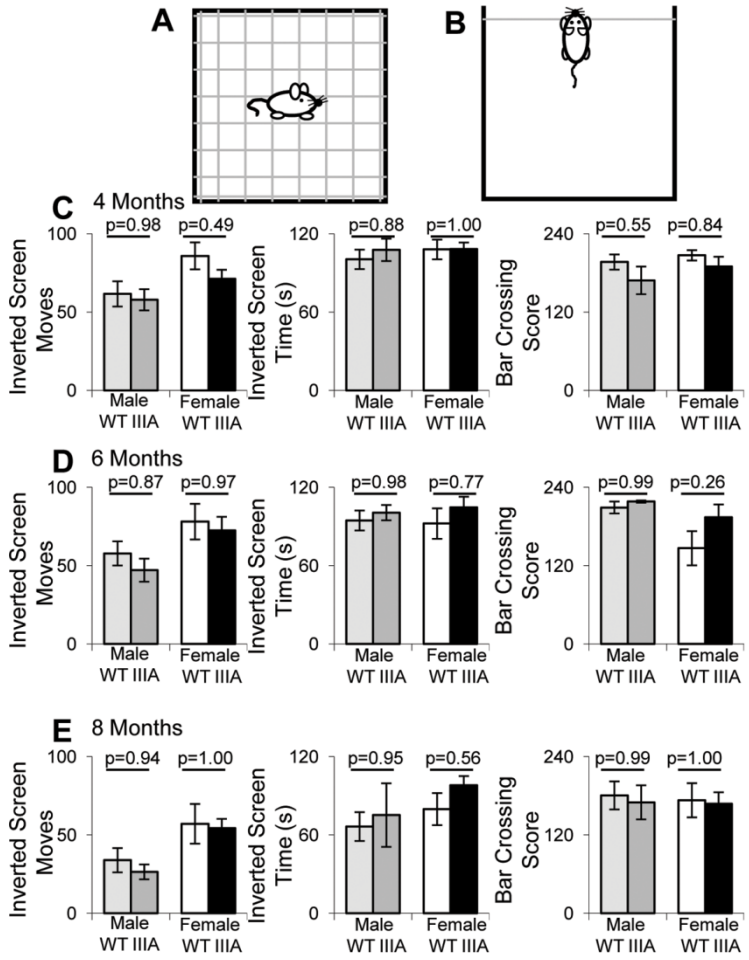

Figure 4-7 Neuromuscular behaviour

At 4, 6 and 8 months of age 8-11 WT male (light grey bars), MPS IIIA male (dark grey bars), WT female (white bars) and MPS IIIA female (black bars) mice were placed on the inverted screen test (A) and horizontal bar crossing test (B). The number of rear leg moves, the time spent on the inverted screen and the bar crossing score are presented at 4 (C), 6 (D) and 8 (E) moths of age. Error bars represent the SEM and p values were calculated by 2 Way ANOVA.

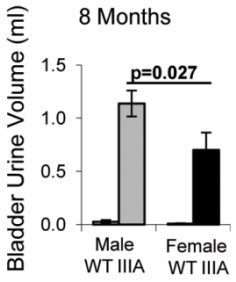

Figure 4-8 Urine retention at 8 months

At 8 months of age 9 WT male (light grey bars), 8 MPS IIIA male (dark grey bars), 7 WT female (white bars) and 8 MPS IIIA female (black bars) were sacrificed and the urine volume in the bladder measured. The results are presented as the mean with error bars representing the SEM. p values were calculated by 2 Way ANOVA.

4.5 Discussion

We have backcrossed the mixed background MPS IIIA mouse model onto the C57BL/6J background and characterised a behavioural phenotype that can be used to accurately distinguish female MPS IIIA mice from WT littermates. Our findings clearly show that male mice show no significant difference to WT littermates in the open field test or elevated plus maze, whilst female MPS IIIA mice demonstrate characteristic hyperactive behaviour initially at 4 months (16 weeks) and more strongly at 6 months (24 weeks), which declines again at 8 months (32 weeks) of age. We also show that these mice have a reduced thigmotaxis at 4 and 6 months of age which would indicate a reduced sense of danger. Hyperactivity and a reduced sense of danger are observed in the behavioural phenotype of the MPS IIIA patients (Cleary *et al.* 1993; Bax *et al.* 1995; Valstar *et al.* 2008; Heron *et al.* 2011).

In this study we observed no significant behavioural differences between WT and MPS IIIA male mice. This is in contrast to Hemsley *et al.* 2005 where hyperactivity was initially observed at 3 weeks, hypoactivity at 6 and 15 weeks and no changes at 10, 20 and 40 weeks in male mixed background mice (Hemsley *et al.* 2005). Lau *et al.* 2008 used male MPS IIIA mice that had been back crossed onto the C57BL/6 background and observed no differences at 3, 5 and 15 weeks, with significant hypoactivity at 10, 18 and 22 weeks (Lau *et al.* 2008) but subsequently observed hypoactivity in 15 week old male mice (Lau *et al.* 2010b). Crawley *et al.,* using male MPS IIIA mice on the same C57BL/6 background, found no changes at 4, 6, 8, 10, 15, 18, 22, 25, 32 and 40 weeks and only observed hyperactivity at 12 weeks (Crawley *et al.* 2006). Finally, using male mice independently crossed onto the C57BL/6J background by Jackson laboratories, hypoactivity was observed in MPS IIIA mice at 20 weeks of age by McIntyre *et al*. but no difference was found at 24 or 32 weeks (McIntyre *et al.* 2008). Clearly, it is hard to obtain consistent behavioural readouts from male mice in this context and it is always difficult to know if hypoactivity could be confounded by any parameter affecting the mouse's physical ability to move. We find male MPS IIIA mice to be very aggressive and are unable to keep them group housed, which is likely to change their behaviour. Singly housing male C57BL/6 mice has been shown to increase locomotor activity and reduce anxiety (Voikar *et al.* 2005) or alternatively in other researchers hands to have no effect

(Arndt *et al.* 2009). At the very least, it certainly leads to weight gains, which will restrict movement, and this may confound reliable open field measures. Additionally we find that all MPS IIIA mice retain urine over time, with male mice retaining significantly more urine at 8 months of age and urine retention is the humane endpoint (Figure 4-8). Clearly urinary retention could be restricting movement which would confound behavioural measures. Lastly, it is worth pointing out that many male mouse studies are on mixed, C57BL/6 or C57BL/6J backgrounds, some of which have been performed in different laboratories, which could lead to inconsistencies in outcomes. Overall, we would argue that comparisons of male MPS IIIA mice with WT are not appropriate (or easy to perform) for determination of treatment responses.

Several studies analysed behaviour of male MPS IIIA mice following control intracranial injections. Fraldi *et al.* tested male mice and found that the MPS IIIA mice were hyperactive at 21 weeks of age but not prior to this, but these mice had received intracranial injections and this may have adversely affected the mouse's behaviour (Fraldi *et al.* 2007). Other studies where intracranial injections have been given to MPS IIIA mice all used male mice and generally observed either no changes or hypoactivity (Hemsley *et al.* 2007; Hemsley *et al.* 2009a; McIntyre *et al.* 2010). It is worth considering that intracranial injections could have altered the natural behaviour of the mouse and this may be why hypoactivity is observed.

We found female MPS IIIA mice to be hyperactive, having a significantly increased path length, frequency and duration of rapid exploratory behaviour and reduced duration of immobility at 4 and 6 months of age, (16 and 24 weeks) whilst duration of rapid exploratory behaviour was significantly increased at 8 months (32 weeks). At 4 months of age differences are only significant after 60 minutes, but at 6 months they are all significant after both 10 and 60 minutes. This indicates that the 60 minute test is a more sensitive test to identify hyperactivity than the 10 or 3 minutes tests. No significant differences were observed over the first 3 minutes at any time point, thus we would suggest that studies such as that of Crawley *et al.* 2006 where this short test has been used are not likely to yield significant differences (Crawley *et al.* 2006).

Our hyperactivity findings in females are supported by Crawley *et al,* where hyperactivity was observed at 22 and 32 weeks of age in a 3 minute test with female MPS IIIA C57BL/6 mice, however no difference was observed at any point prior to 18 weeks or at 25 or 40 weeks of age (Crawley *et al.* 2006). They also observed hypoactivity in females at 4 weeks of age but we do not have comparative data to comment on this. The longer testing time utilised in our study may have meant that we were better able to detect behavioural changes in the mice that were not detectable with the 3 minute test.

On the mixed background, female MPS IIIA mice were hyperactive at 3 weeks of age but at subsequent ages no differences were observed at 6, 10, 15, 20, 30 or 40 weeks of age (Hemsley *et al.* 2005). In the work of Lau *et al*., using the C57BL/6 backcross, no differences were observed between female WT and MPS IIIA C57BL/6 mice between 3-22 weeks of age (Lau *et al.* 2008).

MPS IIIB is a phenotypically indistinguishable disease to MPS IIIA. In the mouse model of MPS IIIB, hyperactivity has been observed in a 10 minute open field test (Cressant *et al.* 2004) but hypoactivity in an 8 minute open field test performed half in the light half in the dark (Li *et al.* 1999). A 60 minute open field test did not report differences in path length in male or female mice (Fu *et al.* 2007), but a 60 minute open field test that we performed on female mice at the same circadian time as this study observed hyperactivity at 8 months of age (Langford-Smith *et al.* 2011b), which is consistent with our findings here. We also observed significant increases in hyperactivity in male MPS IIIB mice at 8 months of age, although they were less significant differences than those observed in females (Malinowska *et al.* 2010). The MPS IIIB mouse is a complete knockout and thus may be slightly more severe than the MPS IIIA mouse with ~3% residual enzyme activity. This may explain why we were able to detect significant genotype differences at 8 months of age in MPS IIIB in all parameters but only some in MPS IIIA. We believe that the 60 minute open field test is still measuring habituation behaviour due to the profiles of activity observed in both MPS IIIA mice in this paper and in MPS IIIB mice (Langford-Smith *et al.* 2011b) over time. We found that differences in behaviour between MPS IIIB and WT mice (Langford-Smith *et al.* 2011b) and MPS IIIA and WT mice (Figure 4-1B

and C) became more pronounced from 3 to 10 to 60 minutes and diverged in the first few minutes from a similar initial response. When we compared MPS IIIB and WT mice over a 24 hour period following 24 hours of habituation (Langford-Smith *et al.* 2011b) the differences were significantly muted and over 14 days there was no significant difference (Canal *et al.* 2010). This suggests that a 60 minute open field test does not reflect home cage behaviour but instead probably measures extended habituation to a novel environment.

A significant decrease in the number of unsupported rears by female MPS IIIA mice was observed at 6 months of age, however, there was no difference in the overall number of rears, or supported rears, at this or any other time point. No consistent trend in the number of rears with female mice is apparent in the literature, Crawley *et al.* reported that female MPS IIIA mice reared less at 15 and 25 weeks of age but there were no significant differences at 9 other time points (Crawley *et al.* 2006). No significant differences in rearing were observed in Lau *et al.* who also used backcrossed mice (Lau *et al.* 2008), or Hemsley *et al* who used mixed background mice (Hemsley *et al.* 2005). A more consistent trend is observed in male mice with MPS IIIA rearing less (Hemsley *et al.* 2005; Crawley *et al.* 2006; Hemsley *et al.* 2007; Lau *et al.* 2008; Hemsley *et al.* 2009a), but increased rearing has been observed at 3 weeks (Hemsley *et al.* 2005). However, at most time points no significant difference is observed. Manual measurement of rears can be subjective and there may be variations in the rearing behaviour, such as amount of time per rear that cannot be measured by simply counting the number of rears. This is why we examined the number of supported and unsupported rears (Benjamini *et al.* 2010; Langford-Smith *et al.* 2011b). In the MPS IIIB mouse model, no difference in rearing was observed in the first 10 minutes of an open field test with female mice at 8 months of age (Langford-Smith *et al.* 2011b) and no difference in the first 30 minutes but a significant decrease in the number of rears by 4.5 - 5 month old male MPS IIIB mice in the second 30 minutes of a 60 minute open field test (Fu *et al.* 2007). Our conclusion is that rearing is too variable an outcome in MPS IIIA and IIIB mice and is therefore not a valuable informative test.

At 4 and 6 months of age the MPS IIIA female mice had a reduced sense of danger and spent a greater proportion of time in the centre of the open field, this measure was significant after 60 minutes but not after 3 or 10 minutes. This is supported by the patients with MPS IIIA which are believed to have a reduced sense of danger (Heron *et al.* 2011). No difference in the time in centre was detected by Lau *et al.* in both male and female mice (Lau *et al.* 2008), however this could be because the open field was larger in our study and therefore more sensitive to thigmotaxis (Crawley 2007; Benjamini *et al.* 2010). In the MPS IIIB mouse model no differences were observed in the duration spent in the centre of the open field (Langford-Smith *et al.* 2011b) but the open field used in that study was also smaller than the one used in this study.

The elevated plus maze did not demonstrate a decrease in anxiety or sense of danger in the MPS IIIA mice. There were also no differences between repeat tests, and both WT and MPS IIIA mice appeared to habituate equally. Lau *et al.* 2010, observed no significant differences with male mice at 6.5 months (26 weeks) of age with a 5 minute elevated plus maze test, but there was a trend towards reduced anxiety (Lau *et al.* 2010a). However in Lau *et al.* 2008 a significant increase in the time the male MPS IIIA mice spent in the open arms at 18 weeks of age, the percentage of the path length on the open arms at 15 and 18 weeks and the percentage of entries into the open arms at 20 weeks was observed (Lau *et al.* 2008). Additionally they observed significant differences between repeats of the elevated plus maze, male MPS IIIA mice had a significantly longer path length in the repeat elevated plus maze test while WT mice remained unchanged (Lau *et al.* 2008). We did not observe decreased anxiety or a change in behaviour between repeat elevated plus maze tests. The width of the arms could be affecting this test as this study used 10 cm wide arms and Lau *et al*., used 7cm wide arms. It has been demonstrated that when using 5, 7 or 9 cm wide arms, NMRI mice spent more time in the wider open arms compared to C57BL/10J mice that showed no difference (Lamberty *et al.* 1996). Ten minutes on the elevated plus maze may also be too short a time to detect the sense of danger differences in the MPS IIIA mice as the only centre measure on open field that is significant after 10 minutes is the number of centre entries in female mice at 6 months of age ($p=0.023$). A lack of difference

in the elevated plus maze performed in daylight is supported by MPS IIIB mice which showed no difference in the light but were less anxious in the dark (Cressant *et al.* 2004). The increased number of centre entries and duration in the open field could perhaps reflect increased but undirected anxiety whereby mice are actually more agitated but have lost the ability to determine what is dangerous. A more pragmatic explanation could be due to the mice not being naively tested as they only received a 30 minute break after the open field test before starting the elevated plus maze test. Prior behavioural tests can affect the result of elevated plus maze tests with C57BL/6J mice that have previously been tested in an open field test (Voikar *et al.* 2004).

No significant differences were observed in the inverted screen or bar crossing tests which is consistent with the work of Crawley *et al.* who observed no difference in neuromuscular strength with back crossed MPS IIIA mice (Crawley *et al.* 2006). However on the mixed background strain, there was a significant decrease in neuromuscular strength with MPS IIIA mice being unable to grip the inverted screen for as long as the WT mice from 20 weeks of age (Hemsley *et al.* 2005). The work in our study is supported by work with the MPS IIIB mouse model that does not show a decline in motor function at 5 months of age by accelerating rotarod (Fu *et al.* 2007; Fu *et al.* 2010) but does by a rocking rotarod test from 9 months onwards (Heldermon *et al.* 2007; Heldermon *et al.* 2010). The decline in motor function could relate to the presence of increasing urine retention in the MPS IIIA and B mouse models (Bhaumik *et al.* 1999; Gografe *et al.* 2009; Malinowska *et al.* 2010). Our own tests in the MPS IIIB mouse model found that neuromuscular decline only occurred at 10 months of age (40 weeks) (Malinowska *et al.* 2010; Langford-Smith *et al.* 2011b). Given that the MPS IIIA mouse has residual enzyme activity, it is possible that progression of MPS IIIA disease is slightly less rapid than in MPS IIIB which would explain why we did not see a trend to changes in motor function at 8 months in MPS IIIA mice.

Standardisation of tests is a very important aspect of behavioural test design that may have affected comparison of our tests to those of other laboratories. In this study the size of the open field was larger than that described in many studies and

the behaviour was observed for much longer (60 minutes compared to 3 minutes). The increased size and time of the test allows better spatial and temporal resolution of the mouse behaviour and so makes the test more sensitive (Benjamini *et al.* 2010). Video analysis software was used which has been shown to be more sensitive than line crossing determined by an observer or beam breaks (Kafkafi *et al.* 2003). In this study, the mouse was placed in the centre of the open field rather than in the corner and the test was carried out at the same time point of circadian rhythm for all mice as there is a known peak in activity in the similar MPS IIIB mouse model (Canal *et al.* 2010). The mice in this study were also housed differently; females were group housed and male mice were singly housed from 14 weeks of age. The background of the mice can affect the behavioural phenotype, therefore we backcrossed the MPS IIIA mice in this study onto the C57BL/6J background for more than 10 generations. Other groups have used MPS IIIA mice on a mixed background (Hemsley *et al.* 2005; Hemsley *et al.* 2007), or on a C57BL/6 (Crawley *et al.* 2006; Fraldi *et al.* 2007; Lau *et al.* 2008; Lau *et al.* 2010a) or C57BL/6J (McIntyre *et al.* 2010) background. Strain specific differences can also significantly affect behavioural outcomes (Gerlai 1996).

4.6 Conclusion

We have demonstrated that we can reliably detect differences in the behaviour of female but not male MPS IIIA mice at 4 and 6 months (16 and 24 weeks) of age and these differences match the patient phenotype. Male mice were singly housed due to their aggression which may change comparative behavioural responses. Female MPS IIIA mice are hyperactive, with a longer path length, increased frequency and duration of rapid exploratory behaviour, and spend less time immobile. They also show a trend to this behaviour at 8 months of age with significantly increased duration of rapid exploratory behaviour. Female MPS IIIA mice demonstrate a reduced sense of danger with a greater proportion of the time spent in the centre of the open field with a greater number of centre entries. These two time points and multiple measures are ideal to evaluate novel therapies for MPS IIIA as the effect of a therapy can be sensitively determined using multiple measures. This

behavioural phenotype is supported by both the phenotype of the patients and our recent similar observations in the MPS IIIB mouse model (Malinowska *et al.* 2010; Langford-Smith *et al.* 2011b). The increased size of the open field, the increased length of the test, the use of video analysis software and performing the test at consistent point in the circadian rhythm have produced a sensitive and robust test to evaluate the effect of therapies on female MPS IIIA mice.

Supplementary Video 4-1 MPS IIIA open field behaviour video

A video running at 4 times the normal speed showing the median female WT and MPS IIIA mouse at 6 months of age. The MPS IIIA mouse is on the left and the WT mouse is on the right. Please see enclosed CD.

Chapter 5 – Stem Cell Gene Therapy For MPS IIIA

Haematopoietic Stem Cell and Gene Therapy Corrects Primary Neuropathology and Behaviour in Mucopolysaccharidosis IIIA Mice Where Conventional Transplant Fails

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5.1 Abstract

Mucopolysaccharidosis type IIIA (MPS IIIA or Sanfilippo disease) is a neurodegenerative disorder caused by a deficiency in the lysosomal enzyme sulphamidase (SGSH), catabolising heparan sulphate (HS). Affected children present with severe behavioural abnormalities, sleep disturbances and progressive neurodegeneration, leading to death in their second decade. MPS I, a similar neurodegenerative disease accumulating HS, is treated successfully with haematopoietic stem cell transplantation (HSCT) but this treatment is ineffectual for MPS IIIA. We compared HSCT in MPS IIIA mice using donor cells transduced *ex vivo* with lentiviral vector expressing SGSH (LV-WT-HSCT) versus wild-type donor cell transplant (WT-HSCT) or autologous transduction (LV-IIIA-HSCT). LV-WT-HSCT results in 10% of normal brain enzyme activity, near normalisation of brain HS and GM2 gangliosides, significant improvements in neuroinflammation and complete behavioural correction. Both WT-HSCT and LV-IIIA-HSCT mediated improvements in GM2 gangliosides and neuroinflammation but were less effective at reducing HS or in ameliorating abnormal HS sulphation observed in MPS IIIA mice, and had no significant effect on behaviour. This suggests that HS has a more significant role in neuropathology than either neuroinflammation or GM2 gangliosides. These data provide compelling evidence for the efficacy of lentiviral mediated HSCT for neurological correction of MPS IIIA where conventional transplant is ineffectual.

5.2 Introduction

Mucopolysaccharidosis IIIA (MPS IIIA or Sanfilippo type A) is a neurodegenerative lysosomal storage disease resulting from a deficiency in the enzyme sulphamidase (N-sulphoglucosamine sulphohydrolase, SGSH, EC 3.10.1.1), caused by mutations in the *SGSH* gene (Valstar *et al.* 2008). The enzyme deficiency leads to accumulation of heparan sulphate (HS) in cells, leading to cellular and organ dysfunction, particularly in the brain (Valstar *et al.* 2008). Patients present with progressive failure to achieve developmental milestones, severe behavioural changes including hyperactivity and sleep disturbances, later cognitive and motor function decline and a markedly shortened lifespan (Meyer *et al.* 2007; Valstar *et al.* 2008; Heron *et al.* 2011). The age of presentation and severity of symptoms varies significantly. Disease neuropathology is poorly understood, with several factors probably contributing to the onset of disease including primary HS storage in the brain, secondary storage of GM gangliosides, amongst other lipids (Bhaumik *et al.* 1999; McGlynn *et al.* 2004) and severe neuroinflammation (Savas *et al.* 2004; Fraldi *et al.* 2007; Arfi *et al.* 2011). There are no current treatments for MPS III.

Intravenous enzyme replacement therapy (ERT) is a successful treatment for attenuated MPS diseases storing HS, such as MPS I Hurler-Scheie, which has limited neurological involvement due to residual enzyme activity in the brain. In this case, delivered recombinant enzyme is taken up by mannose-6-phosphate receptors and cross corrects residual enzyme deficient recipient cells. Since enzyme is unable to cross the blood brain barrier, intravenous ERT is ineffective in neuronopathic MPS diseases including MPS I Hurler (IH) and MPS IIIA.

Patients with MPS IH usually receive haematopoietic stem cell transplantation (HSCT) (Boelens *et al.* 2009; Wynn *et al.* 2009b). Donor cells repopulate the recipient's haematopoietic system and engrafted donor leukocytes secrete enzyme that can cross-correct cells in the periphery. In addition, monocytes traffic from the bone marrow into the brain where they differentiate into microglial cells and mediate cross correction in the recipient central nervous system (CNS) (Krivit *et al.* 1995). As long as treatment is delivered early in life, this results in significant beneficial effects on cognitive outcomes, lifespan, and peripheral bone and joint

disease in MPS IH patients (Shapiro *et al.* 1995; Boelens *et al.* 2009; Wynn *et al.* 2009b).

In contrast, MPS IIIA patients show increased lifespan but no significant neurological improvements after HSCT, despite storage of very similar substrates in the brain (Shapiro *et al.* 1995; Sivakumur *et al.* 1999; Prasad *et al.* 2008). Following unrelated cord blood transplants, one year patient survival rates are similar (77% MPS IH, 79% MPS III) but 3 year patient survival is markedly different (75% MPS IH, 56% MPS III), suggesting that engraftment is successful but that transplant is not curative for MPS III (Prasad *et al.* 2008). We have recently reported that metabolic correction, (expressed as reduction of glycosaminoglycan substrate), of MPS I patients receiving transplants from heterozygote donors with one enzyme gene copy, is less complete than those receiving unrelated transplants from homozygous donors with two enzyme gene copies (Wynn *et al.* 2009b). HSCT failure in MPS IIIA patients could therefore be due to insufficient enzyme being produced by donor-derived microglia in the brain (Shapiro *et al.* 1995; Sivakumur *et al.* 1999), whilst gene therapy could be an approach to increase secreted enzyme in the brain beyond that achieved by wild type transplantation.

A clinically relevant gene therapy approach for MPS IIIA and the clinically indistinguishable MPS IIIB, is direct brain delivery of AAV (Cressant *et al.* 2004; Fraldi *et al.* 2007; Heldermon *et al.* 2010). However, this approach is very invasive and has potential scale-up issues with limited distribution of vector from the injection sites in the brain (Wang *et al.* 2003; Taymans *et al.* 2007), as well as the potential for immune responses in patients exposed directly to vector or enzyme (Worgall *et al.* 2008).

The alternative approach of *ex vivo* gene delivery to HSCs, using a lentiviral vector (LV-HSCT), has become progressively more clinically achievable for neurodegenerative metabolic diseases in recent years. This is due to vastly improved HSCT survival rates, of over 90% for MPS IH (Boelens *et al.* 2009), and several studies showing the potential for correction of neurodegenerative diseases via HSC modification (Biffi *et al.* 2006; Cartier *et al.* 2009; Gentner *et al.* 2010; Visigalli *et al.* 2010). *Ex vivo* LV-HSCT was used to replace the arylsulphatase A

enzyme in a mouse model of metachromatic leukodystrophy, and achieved 10% of normal brain enzyme and neuronal correction (Biffi *et al.* 2006), which has resulted in an ongoing clinical trial. In MPS I, erythroid specific LV-HSCT resulted in neurological correction of mice (Wang *et al.* 2009), whilst another LV-HSCT approach has resulted in 5 fold increases in brain enzyme and significant improvements in peripheral disease in MPS I mice (Visigalli *et al.* 2010). In mouse models of MPS IIIA and IIIB, HSCT alone is unable to correct the neurological phenotype (Heldermon *et al.* 2010; Lau *et al.* 2010a). However, a retroviral HSCT approach in MPS IIIB mice resulted in 25% of normal brain enzyme activity in two cases; although copy numbers were not stated and behavioural analysis was not performed (Zheng *et al.* 2004). No data are published on LV-HSCT in MPS IIIA.

To evaluate the efficacy of LV-HSCT against normal HSCT in MPS IIIA mice we compared MPS IIIA mice receiving either WT or MPS IIIA cells that were *ex vivo* transduced with an *SGSH* expressing lentiviral vector, against mice receiving normal HSCT. We have demonstrated that LV-HSCT can significantly increase the SGSH enzyme activity in the brain, normalise brain HS, reduce secondary pathology and correct behaviour of MPS IIIA mice where normal HSCT fails.

5.3 Methods

5.3.1 Construction and Testing of SGSH Lentiviral Vector

The lentiviral vector plasmid pHR'SIN-cPPT-SEW (Demaison *et al.* 2002) was modified by replacing the eGFP gene with a Gateway conversion cassette (Invitrogen Life Technologies #11828-019) containing two attB sites flanking a *ccdB* positive selection gene, producing pHRsin.SFFV.Gateway. This produced a lentiviral backbone that could act as a Gateway destination vector so that genes could be cloned in directly using recombination technology. The human SGSH cDNA sequence including 5' and 3' UTR from image clone #5226903 (Geneservice) was Gateway cloned into a donor vector (pDonor221) containing attP sites as described (Hartley *et al.* 2000) with BP Clonase (Invitrogen). This resulted in SGSH cDNA being flanked by attL sites enabling Gateway cloning of SGSH cDNA into the lentiviral

vector using LR Clonase to create pHRsin.SFFV.hSGSH.att.wpre. To confirm the ability of microglia to produce active enzyme from the construct, the human microglial cell line CHME3 was transfected with pHRsin.SFFV.hSGSH.att.wpre using Fugene 6 as per manufacturer's instructions (Roche). Three days later, media and cells were analysed for SGSH activity.

5.3.2 Lentiviral Vector Production and Titration

HEK 293T cells were seeded in 15cm tissue culture plates (Corning) in DMEM/10%FCS/2mM L-glutamine, (Lonza) and cultured overnight at 37°C until 40-70% confluent. VSV-G pseudo-typed lentiviral vector was produced by the transient transfection of HEK 293T cells with pHRsin.SFFV.hSGSH.att.wpre, pMD2G and pΔ8.91gag/pol (Bigger *et al.* 2006) in a 3:1:2 ratio using a total of 15µg of plasmid DNA per dish, 44.5µl of Fugene 6 (Roche) in Optimem (Gibco) as per manufacturer's instructions. The media was replaced 4 hours and 24 hours after transduction and harvested at 36 and 60 hours. Cells were removed by centrifugation at 262g for 15 minutes at 4°C, and filtered through a 0.45µm low protein binding filter (Nalgene). Lentiviral vector particles were concentrated by centrifugation at 21,191g for 150 minutes at 4°C, resuspended in PBS and stored at -80°C. Lentiviral vector was titred using a similar method to Kutner *et al.* (Kutner *et al.* 2009). $1x10^5$ murine lymphoma (EL4) cells (ATCC Number TIB-39, Sigma) were cultured in RPMI 1640 (Lonza)/10% FCS/2mM L-glutamine and transduced with 5 dilutions of concentrated lentiviral vector. Four days later, genomic DNA was extracted from the cells using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma Aldrich) and analysed by Quantitative Real Time PCR (QPCR) to determine the number of integrated lentiviral genomes per cell. The infectious titre was calculated as the number of cells at transduction multiplied by the number of lentiviral copies per cell divided by the volume of lentiviral vector added. Titres of $1.1x10^9$TU/ml (integrated transducing units) were normally reached.

5.3.3 QPCR Copy Number Determination

The WPRE copy number of lentiviral vectors in genomic DNA from murine cell lines, bone marrow, blood and spleen samples was determined by QPCR using the Applied Biosystems 7500 Real Time PCR System. A primer and probe set against

wpre (TAMRA) were used as previously described (Lizee *et al.* 2003) and standardised against rodent *gapdh* primer and probe set (VIC) (Applied Biosystems). Samples were run in duplicate 25µl reactions using the cycling parameters 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The copy number was determined using a standard curve generated by dilutions of genomic DNA from an EL4 cell line clone (ALS EL4 eGFP 2,2) containing 2 integrated copies/cell of pHRsin.SFFV.eGFP.att.wpre. The EL4 cell clone (ALS EL4 eGFP 2,2) was made by transducing EL4 cells with the pHRsin.SFFV.eGFP.att.wpre lentiviral vector expressing eGFP, followed by two consecutive rounds of single cell cloning. The lentiviral copy number per cell was determined by Southern blot of whole genomic DNA as previously described (Themis *et al.* 2005).

5.3.4 Mice and Transplant Procedures

All *in vivo* procedures were ethically approved in accordance with UK Home Office regulations. MPS IIIA mice (Bhaumik *et al.* 1999) were backcrossed for 10 generations onto the C57BL/6J background (B6.Cg-Sgsh^{mps3a}/6J), maintained by heterozygote breeding and genotyped as previously described (Langford-Smith *et al.* 2011a). Littermate controls were used throughout. MPS IIIA mice were also backcrossed onto the PEP3 CD45.1 congenic background (B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ) to distinguish donor and recipient cells.

Total bone marrow mononuclear cells were isolated from femur, tibia, and ilium of WT CD45.1 or MPS IIIA/CD45.1 mice and lineage depleted using the murine hematopoietic progenitor enrichment cocktail (Stem Cell Technologies) according to manufacturer's instructions. Cells were resuspended in X-Vivo10 (BioWhittaker) and transduced as previously described (Siapati *et al.* 2005).

6-8 week old mice housed in individually ventilated cages were myeloablated with 125mg/kg Busulfan (Busilvex; Pierre Fabre) in 5 daily doses via intra-peritoneal injection. 1 week prior to myeloablation and for a further 7 weeks, mice received acidified water (pH 2.8), irradiated food, mash and sugar free jelly to prevent gastrointestinal infections and to encourage fluid uptake. Within 24 hours of the

final injection of Busulfan, 1.5-2.5x10^5 lineage depleted transduced (LV-SGSH) or un-transduced haematopoietic stem cells were delivered intravenously.

5.3.5 Flow Cytometry

Haematopoietic engraftment was assessed at 6-16 weeks post-transplant in peripheral blood by staining with anti-mouse CD45.1-PE or CD45.2-FITC (BD Pharmingen) in a 5% solution of ToPro3 Iodide (Molecular Probes) on a BD FACS Canto II flow cytometer.

5.3.6 Behaviour

At 4 and 6 months (16 and 24 weeks) of age 10 female mice were assessed for open field behaviour over 60 minutes at the same circadian time point as previously described (Malinowska *et al.* 2010; Langford-Smith *et al.* 2011a; Langford-Smith *et al.* 2011b) and data analysed using TopScan suite software version 2.0 (Clever Sys. Inc).

5.3.7 Sample Processing

At 8 months of age, anesthetised mice were trans-cardially perfused with 37°C Tyrode's. Brains were removed; one hemisphere frozen at –80°C and one fixed in 4% paraformaldehyde for 24 hours then 30% sucrose 2mM $MgCl_2$/PBS for 48 hours before freezing at –80°C. For biochemical assays the snap frozen hemisphere was homogenized so that all assays represent an average of each hemisphere. Liver and spleen were frozen at –80°C. For SGSH and GAG assays, samples of brain, liver or spleen were homogenized and sonicated in homogenisation buffer (0.5M NaCl, 0.02M Tris pH 7-7.5), then centrifuged at 2,200g for 15 minutes at 4°C and the supernatant collected. Protein concentration was determined using the BCA (Thermo Scientific) assay according to manufacturer's instructions.

5.3.8 SGSH Enzyme Activity

SGSH enzyme activity was measured in a two-step protocol using a fluorescent substrate MU-αGlcNS (Moscerdam) as per manufacturer's instructions (Karpova *et al.* 1996) with minor modifications. The amount of starting material was standardised to 40µg of total protein for liver and spleen and 60µg for brain.

5.3.9 Blyscan for Total Sulphated Glycosaminoglycans

The total amount of sulphated glycosaminoglycans (GAG) in 100µg of liver and brain was determined using the Blyscan Kit (Biocolor Ltd.) (Malinowska *et al.* 2009). Actinase E (200µg) was added to the sample, incubated at 55°C for 20 hours before heating at 100°C for 5 minutes and centrifuging at 3,000g for 10 minutes. The supernatant was incubated with 1,9-dimethymethylene blue for 30 minutes with vigorous shaking. After centrifuging at 10,000g for 15 minutes at 4°C the pellet was incubated with dye dissociation reagent for 15 minutes and the colour quantified using a spectrophotometer at 656nm. The quantity was determined against a known concentration GAG standard.

5.3.10 AMAC-labelled Disaccharide Analysis of Heparan Sulphate

Analysis of the quantity and sulphation state of HS was determined as previously described (Holley *et al.* 2011). Total HS was calculated by summing the peak area and applying a labelling efficiency correction factor as described (Holley *et al.* 2011). Three randomly assigned mice from each group were analysed.

5.3.11 Immunohistochemistry

Four brain sections per mouse taken from bregma 0.26, -0.46, -1.18 and -1.94mm were stained for Isolectin B4 and GM2 gangliosides and quantified as previously described (Canal *et al.* 2010; Malinowska *et al.* 2010). Isolectin B4 stained sections were counterstained with Mayer's haematoxylin before mounting. A section from -0.84mm relative to bregma (2 mice per group) was stained for lysosomal associated membrane protein 2 (LAMP2) to demonstrate the size of the lysosomal compartment. Sections were blocked in 5% goat serum, 1mg/ml BSA 0.1% Triton X-100 in TBS for 1 hour and incubated overnight at 4°C with rat anti-LAMP2 IgG (2µg/ml; developed by August, JT, Developmental Studies Hybridoma Bank, University of Iowa, USA) in blocking solution, washed 4x in TBS and stained with Alexa 488 goat anti-rat IgG (1:1000, Invitrogen) in blocking solution for 1 hour in the dark. After washing with TBS, sections were counterstained with 300nM DAPI (Invitrogen) for 15 minutes. Sections were mounted using ProLong Gold Anti-fade mounting medium (Invitrogen). Representative sections from cortex layer IV/V have been displayed.

5.3.12 Statistical Analysis

JMP software version 8 (SAS Institute Inc.) was used with one way ANOVA and Tukey *post hoc* test to analyse data. Data were log transformed where they failed normality tests. Significance was assumed for probabilities of 0.05 or lower. Survival analysis was performed in SPSS version 19 (IBM) using Kaplan-Meier analysis with Mantel-Cox log rank pairwise comparisons.

5.4 Results

5.4.1 Lentiviral HSCT Results in Significant Expression, Transduction and Donor Chimerism

In order to evaluate if *ex vivo* transduction of HSCs using a lentiviral vector expressing SGSH (LV-SGSH) could improve treatment outcomes in MPS IIIA mice, we constructed a LV-SGSH vector driven by the spleen focus forming virus (SFFV) promoter (Figure 5-1A).

To confirm that microglia could over express SGSH without toxicity, the human microglial cell line, CHME3, was transfected with the lentiviral plasmid containing *SGSH*. A 4.5 fold increase in cellular SGSH activity (Figure 5-1B) and an 18 fold increase in secreted SGSH activity (Figure 5-1C) were observed without cellular toxicity.

WT or MPS IIIA lin- bone marrow was either un-transduced or transduced with LV-SGSH before transplant into Busulfan myeloablated 6-8 week old MPS IIIA mice (n=16 per group) (Figure 5-1A). Age matched control groups of WT to WT transplant, un-transplanted WT and MPS IIIA were used for comparison (n=16 per group). At 16 weeks post-transplant, peripheral blood chimerism was 88%, 89% and 90% in the LV-WT-HSCT, LV-IIIA-HSCT and WT-HSCT groups respectively (Figure 5-1D). The average copy number was 0.27 integrations per white blood cell for LV-WT-HSCT and 0.22 integrations for LV-IIIA-HSCT groups (Figure 5-1E), however the copy number for the LV-IIIA-HSCT biochemistry/histology group was only 0.11 copies/cell and for the LV-IIIA-HSCT survival group was 0.34. No adverse events or leukemic blasts have been observed on blood smears from all mice including the survival cohort, kept for over one year post-transplant (n=6 per group).

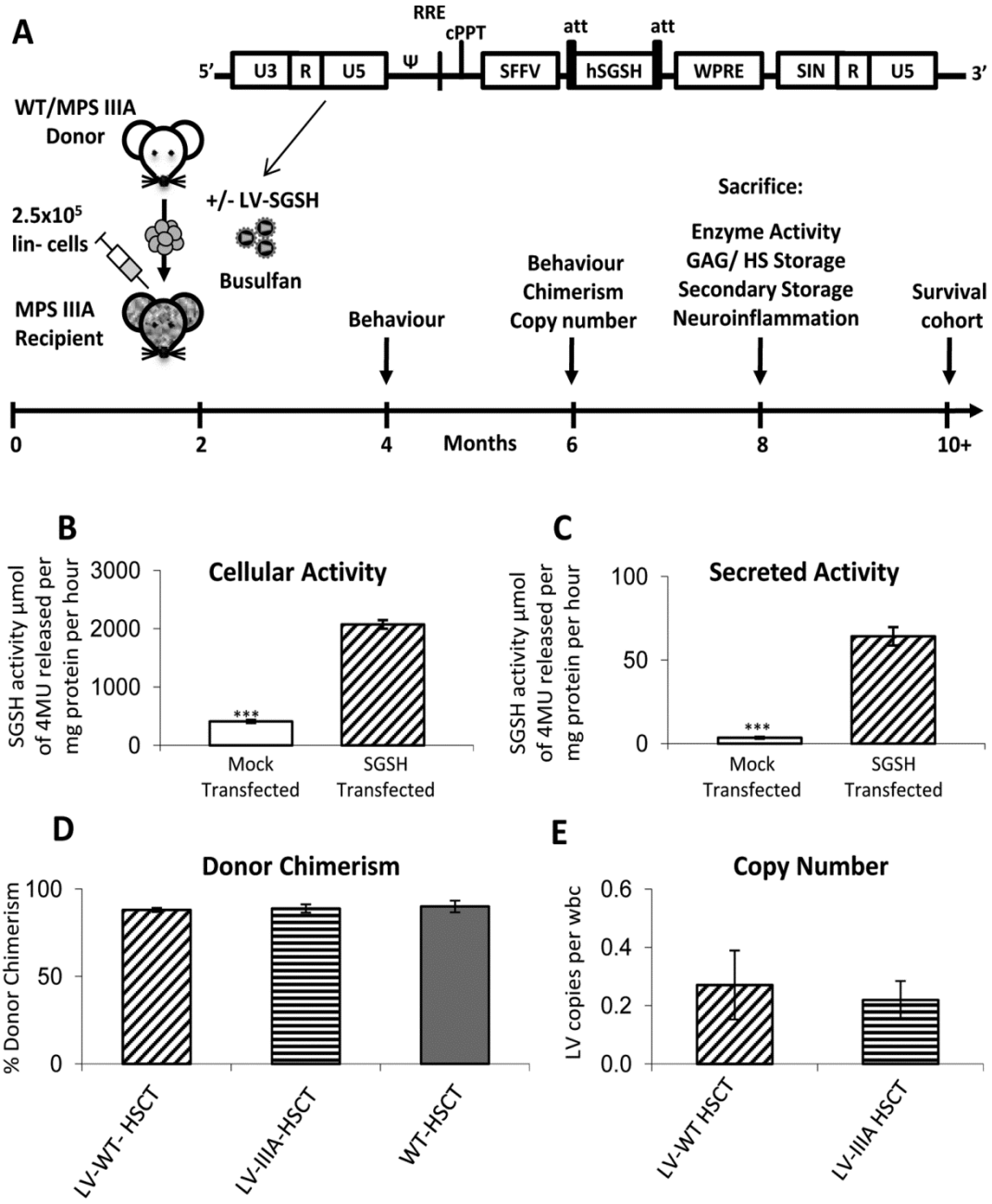

Figure 5-1 Lentiviral SGSH transduced microglia have improved SGSH activity

(A) Lineage depleted bone marrow was transduced or untransduced with a lentiviral vector expressing human *SGSH* under the SFFV promoter and transplanted into Busulfan conditioned recipients at 2 months (8 weeks) of age. Groups were; WT to WT transplant, WT untreated, MPS IIIA untreated, WT donor cells transduced with LV-SGSH into MPS IIIA recipients (LV-WT-HSCT), MPS IIIA donor cells transduced with LV-SGSH into MPS IIIA recipients (LV-IIIA-HSCT) and WT to MPS IIIA (WT-HSCT) (n=16 per group). An open field behavioural test was performed at 4 and 6 months (16 and 24 weeks) of age, chimerism and copy number were determined at 6 months of age, and at 8 months of age (32 weeks of age, 24 weeks post transplant), mice were sacrificed for biochemical and histological analysis and a cohort was kept for survival and sacrificed at their humane endpoint. (B) The human microglial cell line CHME3 was transfected or mock-transfected with LV-*SGSH* and enzyme activity measured in cells and, (C) media. (D) Donor chimerism was determined using flow cytometry at 16 weeks post-transplant. (E) Lentiviral vector copy number in white blood cells was determined by QPCR at 16 weeks post-transplant. Error bars represent the standard error of the mean (SEM) and significant difference is demonstrated with *= $p<0.05$, **= $p<0.01$ and ***= $p<0.001$.

5.4.2 LV-WT-HSCT Increases Brain SGSH and Normalises HS Storage in the MPS IIIA Brain

To determine the outcome of therapy on primary neuropathology, 5 mice per group were sacrificed at 8 months of age and perfused with Tyrode's to flush blood from organs. SGSH enzyme activity was measured in the spleen, liver and brain (Figure 5-2A-C). Untreated MPS IIIA mice expressed approximately 3% of WT activity (Bhaumik *et al.* 1999). All treatments significantly increased SGSH activity in the spleen to 87%, 77% and 53% of WT activity by LV-WT-HSCT, LV-IIIA-HSCT and WT-HSCT respectively. In the liver, SGSH activity was significantly increased to 46%, 32% and 37% of WT activity by LV-WT-HSCT, LV-IIIA-HSCT and WT-HSCT respectively. In the brain, SGSH activity was significantly increased to 10% of WT activity ($p=0.007$) by LV-WT-HSCT and 7% of WT activity ($p=0.05$) by LV-IIIA-HSCT. WT-HSCT increased activity to 6% of WT activity, but this was not significantly elevated over residual MPS IIIA brain enzyme activity of 3%.

Total sulphated glycosaminoglycans (GAGs), including HS, were measured in the liver and brain (Figure 5-2D,E). In the liver, GAGs were normalised to WT levels by LV-WT-HSCT and WT-HSCT, with near normalisation by LV-IIIA-HSCT. In the brain, LV-WT-HSCT and LV-IIIA-HSCT both normalised GAGs to WT levels, whilst WT-HSCT significantly reduced GAG levels. Total brain HS levels were measured following AMAC-labelled disaccharide analysis (Figure 5-2F), revealing that all transplants significantly decreased total brain HS. WT-HSCT and LV-IIIA-HSCT both decreased HS storage by 51%, whereas LV-WT-HSCT normalised brain HS levels to those of WT with a 77% reduction.

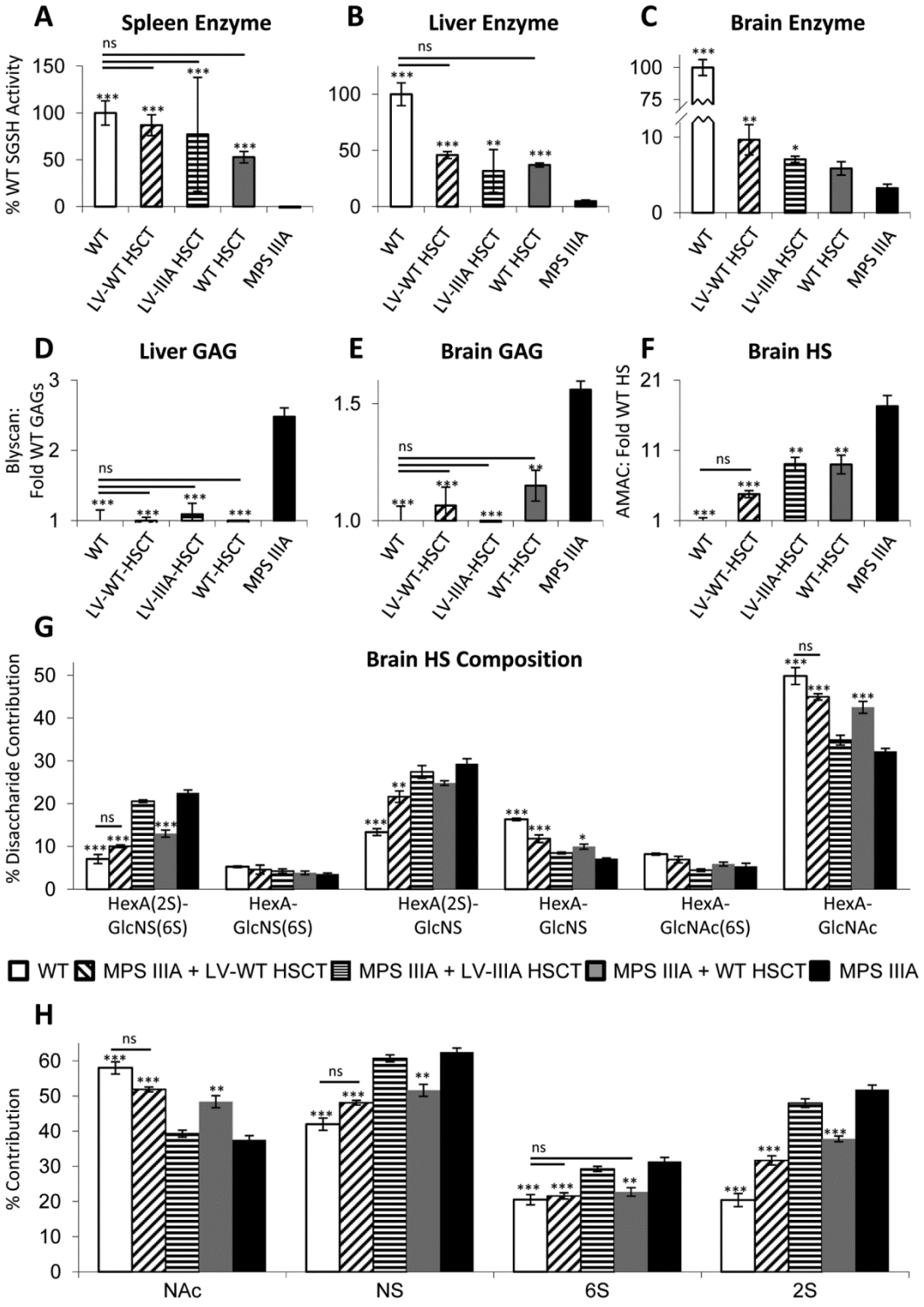

Figure 5-2 LV-WT-SGSH improves enzyme activity and reduces primary storage in MPS IIIA mice

SGSH enzymatic activity was measured in (A) spleen, (B) liver and (C) brain. The level of sulphated glycosaminoglycans was determined using the Blyscan assay in the (D) liver and (E) brain. (F) The level of HS storage in the brain was determined by AMAC. (G) The different HS disaccharides were quantified. (H) The relative proportion of HS that was N-acetylated (NAc), *N*-sulphated (NS), 6-*O*-sulphated (6S) and 2-*O*-sulphated (2S) was also determined by AMAC. Error bars represent the SEM. Significant difference to MPS IIIA is demonstrated with *= $p<0.05$, **= $p<0.01$ and ***= $p<0.001$. Where treatments result in significant improvement to MPS IIIA and there is no significant difference to WT, this is shown by a line and ns.

5.4.3 HS Sulphation is Significantly Increased in MPS IIIA and Normalised by LV-WT-HSCT

Six characteristic disaccharides are revealed by AMAC analysis (Figure 5-2G). Overall, MPS IIIA mouse brains displayed significant increases in the four most highly sulphated disaccharides HexA(2S)-GlcNS(6S) ($p=5.6x10^{-7}$) and HexA(2S)-GlcNS ($p=7.8x10^{-5}$), and commensurate reductions in HexA-GlcNS ($p=1.2x10^{-5}$) and HexA-GlcNAc ($p=2.7x10^{-5}$). This led to overall significant increases in *N*- (p=0.00002), 2-*O*- ($p=4.3x10^{-7}$) and 6-*O*-sulphation (p=0.0007) and a commensurate reduction in *N*-acetylated (unsulphated, p=0.00002) regions over WT (Figure 5-2H). LV-IIIA-HSCT was unable to change this abnormal profile, whilst WT-HSCT significantly improved outcomes in 3 out of 4 abnormal disaccharides (p<0.03-0.00001) and LV-WT-HSCT further improved all 4 abnormal disaccharide profiles ($p<0.01-1x10^{-6}$) and normalised HexA(2S)-GlcNS(6S) and HexA-GlcNAc to WT proportions (Figure 5-2G). Both WT-HSCT and LV-WT-HSCT significantly improved abnormal HS sulphation patterning towards normal WT levels, but only LV-WT-HSCT was able to fully normalise this in the case of NAc, *N*- and 6-*O*-sulphation to WT levels (p=0.0001, p=0.0001, p=0.0006, Figure 5-2H). 2-O-sulphation remained significantly increased over WT (p=0.002). LV-IIIA-HSCT had no significant effect on HS sulphation patterning.

5.4.4 LV-WT-HSCT and WT-HSCT Reduce Secondary Storage and Neuroinflammation Equally

In order to determine if treatment had an effect on lysosomal swelling, we stained the cerebral cortex for LAMP2 (green) and a nuclear stain, DAPI (blue) (Figure 5-3A). MPS IIIA mice exhibited cells with large, dense LAMP2 staining around the nucleus, whilst WT mice displayed discrete punctate LAMP2 staining in a perinuclear location. LV-IIIA-HSCT did not appear to have an effect on abnormal MPS IIIA lysosomal morphology whilst WT-HSCT was able to reduce some of this pathology and LV-WT-HSCT resulted in cells with substantial reduction of abnormal LAMP2 staining.

Severe neuroinflammation in MPS IIIA mice is measurable by the significant infiltration of Isolectin B4 positive microglial cells into the cerebral cortex (Figure 5-3B,D). WT mice have virtually no microglia in this location. Microglial numbers were significantly reduced by 61% in LV-WT-HSCT ($p=1x10^{-7}$), 50% in LV-IIIA-HSCT ($p=3x10^{-6}$) and 61% in WT-HSCT ($p=1x10^{-7}$), but there was no significant difference between treatments and all remained significantly elevated above WT.

MPS IIIA mouse brains also displayed significant secondary storage of GM2 gangliosides, particularly in lamina II/III and V/VI of the primary motor, somatosensory and parietal areas of the cerebral cortex (Figure 5-3C,E) whilst no staining was detected in these areas in WT mice (McGlynn *et al.* 2004). A significant decrease in GM2 gangliosides of 76% (p=0.02) was detected after LV-WT-HSCT and 78% (p=0.02) after WT-HSCT, normalising GM2 gangliosides to WT levels. Treatment with LV-IIIA-HSCT did not significantly reduce GM2 gangliosides but resulted in an average of 10% reduction. However, there was significant variability between mice with some almost completely corrected and others hardly corrected at all (Figure 5-3C). More effective GM2 ganglioside clearance correlated with higher lentiviral copy number. GM2 ganglioside storage was observed in the amygdala, but did not appear to be reduced by any treatment (Figure 5-4).

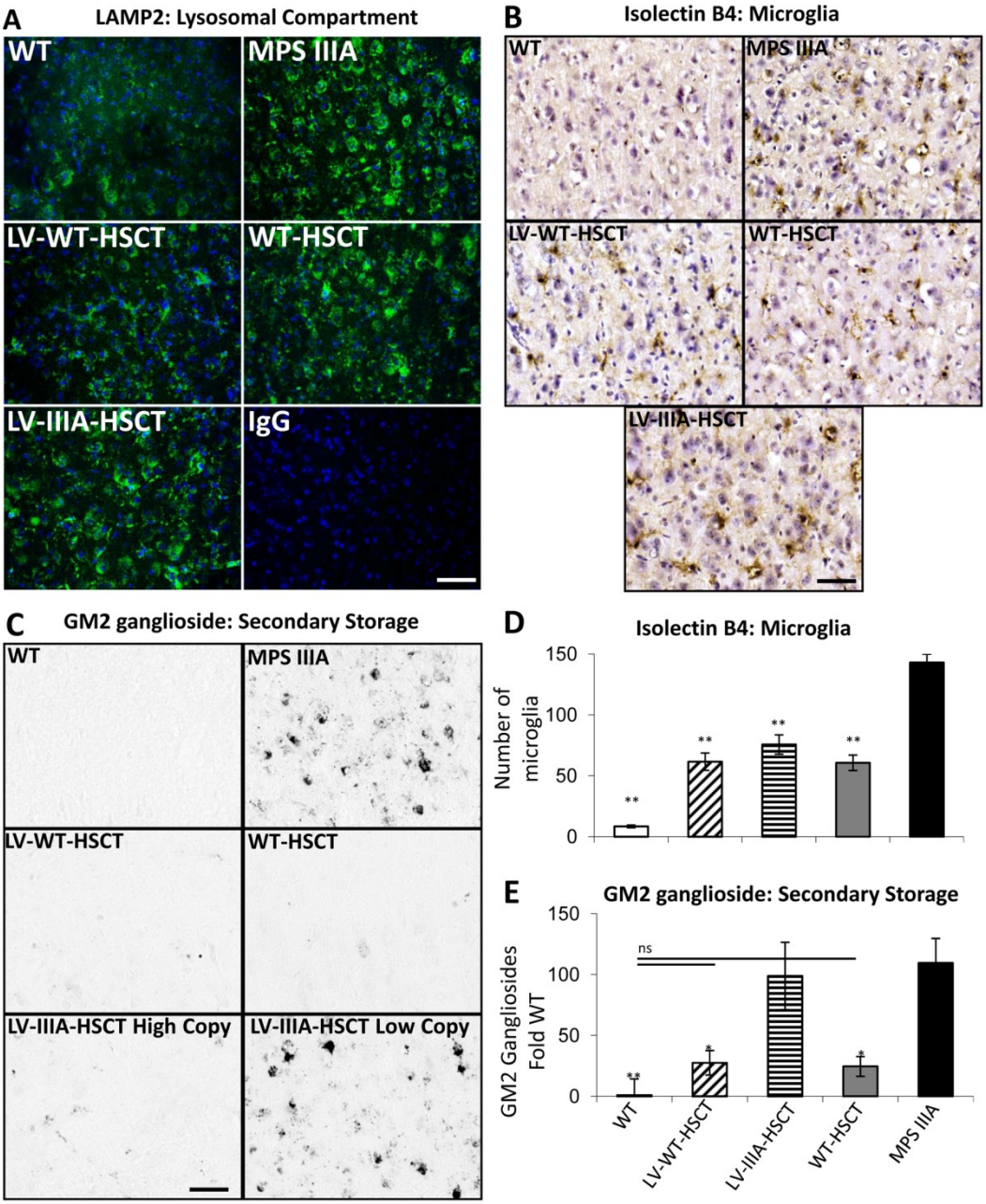

Figure 5-3 Neuropathology is improved by all treatments

(A) Representative sections of brain cortex (layer IV/V) from -0.84mm relative to bregma were stained with LAMP2 (green) to demonstrate lysosomal compartment size and DAPI (blue) to highlight nuclei. (Bar=50µm). (B) Representative images of cerebral cortex layer IV/V from around -0.46mm relative to bregma showing isolectin B4-positive microglia (brown) and nuclei (blue) and (C) GM2 gangliosides (black). Two images of LV-IIIA HSCT are shown, one with a high copy and one with a low copy to demonstrate the variable response in this group (Bar=50µm). (D) The number of microglia were counted and, (E) GM2 ganglioside storage was quantified using Image J from 2 fields of view (x20 objective) per brain section, 4 sections per mouse (n=5 mice per group). Error bars represent the SEM. Significant difference to MPS IIIA is demonstrated with *= $p<0.05$, **= $p<0.01$ and ***= $p<0.001$. Where treatments result in significant improvement to MPS IIIA and there is no significant difference to WT this is shown by a line and ns.

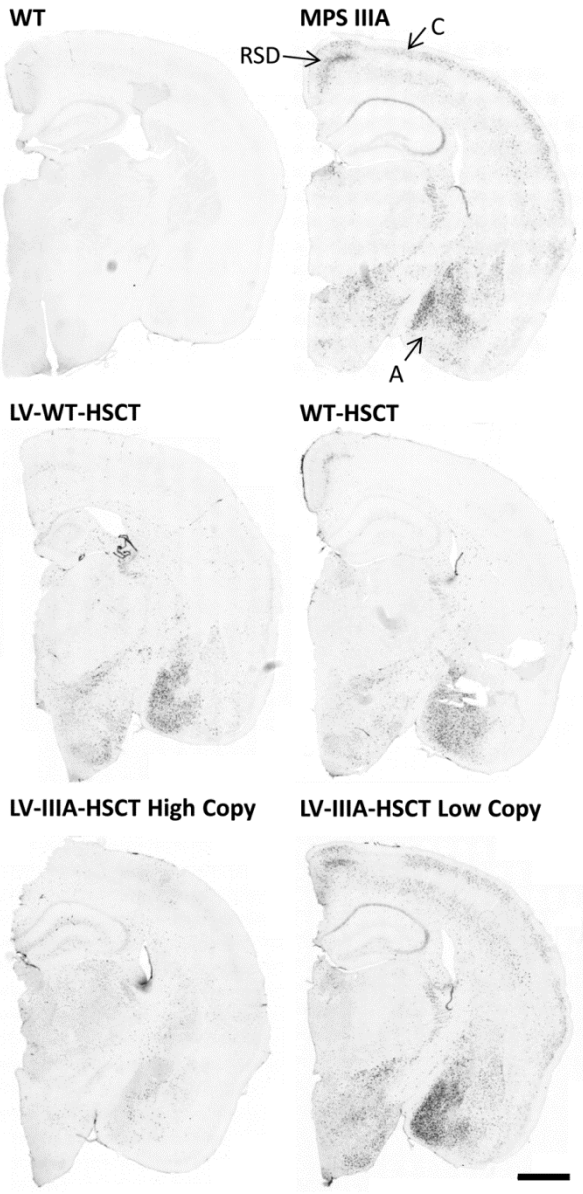

Figure 5-4 GM2 ganglioside storage in whole brain.

Brain sections from approximately -1.18 to -1.94mm relative to bregma stained for GM2 ganglioside (black). The cortex (C), amygdala (A) and retrosplenial granular cortex (RSD) are marked. Bar = 1000μm

5.4.5 MPS IIIA Behaviour is Fully Corrected by LV-WT but Unchanged by WT or LV-IIIA -HSCT

At 4 and 6 months of age a 60 minute open field test was performed to determine the effect of treatment on mouse behaviour (Langford-Smith *et al.* 2011b). In order to control for the effect of Busulfan conditioning we initially compared age matched untransplanted WT mice with WT to WT transplanted mice, and found their behaviour to be indistinguishable (data not shown). In our hands, MPS IIIA mice

present a phenotype of hyperactive behaviour with increased path length, duration and frequency of rapid exploratory behaviour, whilst immobility time is reduced. MPS IIIA mice also displayed reduced thigmotaxis which we interpret as a reduced sense of danger, with increased frequency and duration of centre entries (Langford-Smith *et al.* 2011a). At 4 months of age, MPS IIIA mice showed increased hyperactive behaviour with significantly increased path length (p=0.034, Figure 5-4A) but no significant change in duration or frequency of rapid exploratory behaviour or immobility (Figure 5-5 B,C,D). Frequency and duration of centre entries are significantly increased at 4 months (p=0.02, p=0.03, Figure 5-5E,F). Although no significant reductions are seen with any treatment at this age, LV-IIIA-HSCT and WT-HSCT appear to have little effect on behaviour whilst LV-WT-HSCT shows a clear trend towards normalisation of every parameter to WT levels except centre entry duration.

At 6 months of age, MPS IIIA behaviour diverges further from the WT control mice to give highly significant differences in path length (Figure 5-5G, p=0.002), duration (Figure 5-5H, p=0.001), and frequency of speed over 100mm/s (Figure 5-4I, p=0.002), immobility time (Figure 5-5J, p=0.02), frequency (Figure 5-5K, p=0.001), and duration of centre entries (Figure 5-5L, p=0.04). These behaviours can be observed in Supplementary Video 5-1 and strongly suggest increases in hyperactivity and a reduced sense of danger.

Neither LV-IIIA-HSCT nor WT-HSCT were able to significantly improve any of these abnormal behaviours, although WT-HSCT did show a non-significant trend towards normalisation in sense of danger measures (frequency and duration of centre entries, Figure 5-5K,L). Hyperactivity in particular, was not corrected by either treatment.

In contrast LV-WT-HSCT significantly improved path length (Figure 5-5G, p=0.01), duration (Figure 5-5H, p=0.004), and frequency of speed over 100mm/s (Figure 5-5I, p=0.009), immobility time (Figure 5-5J, p=0.04), and frequency of centre entries (Figure 5-5K, p=0.007), with a non-significant trend to reduction of centre entries (Figure 5-5L, p=0.11). All behaviours were normalised to WT levels and none were significantly different from the WT group.

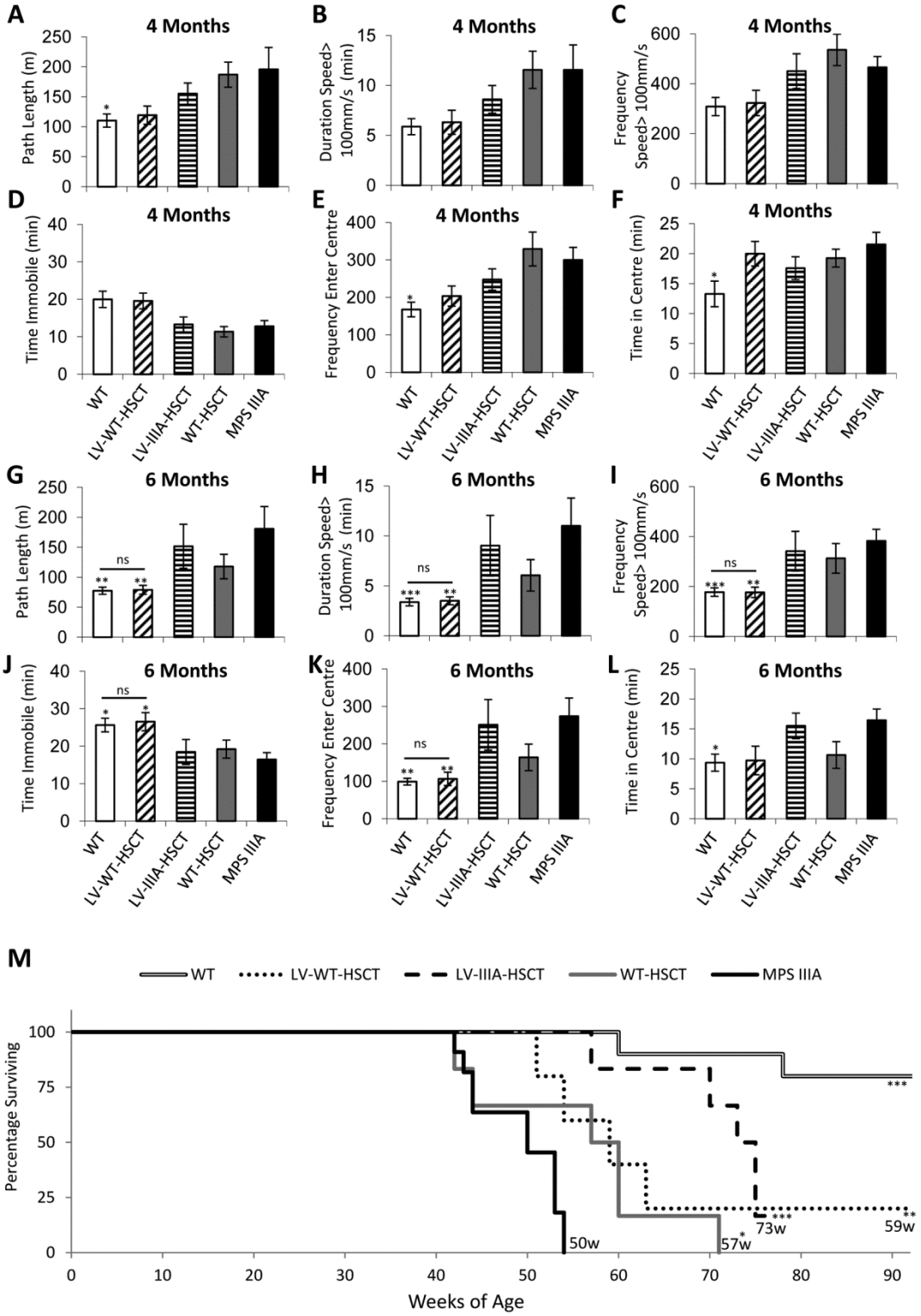

Figure 5-5 Behavioural correction and survival

The open field behaviour test was performed for one hour at the same point of the circadian rhythm at 4 and 6 months (16 and 24 weeks) of age (n=10 female mice per group). The measures of hyperactivity at 4 months were, (A) path length, (B) duration spent moving over 100mm/s, (C) frequency spent moving over 100mm/s, (D) duration immobile, whilst (E) frequency of centre entries and (F) duration in centre measures thigmotaxis and may be a measure of sense of danger. The same parameters, (G-L) were also measured at 6 months of age. (M) A cohort of 6-10 mice from each group were kept to 92 weeks of age. Mice were sacrificed when they reached their humane end point, frequently caused due to urine retention. The median survival in weeks (w) for each group is shown at the bottom of the curve. Error bars represent the SEM. Significant difference to MPS IIIA is demonstrated with *= p<0.05, **= p<0.01 and ***= p<0.001. Where treatments result

in significant improvement to MPS IIIA and there is no significant difference to WT this is shown by a line and ns.

A cohort of 5-10 randomly selected mice per group were kept to assess the effect of treatment on longevity (Figure 5-5M). WT and WT-WT transplanted groups were pooled as survival is not significantly affected in this group. Two WT mice reached the humane end point by week 92, the end of the study. Untreated MPS IIIA mice all died between weeks 42-54. WT-HSCT treated mice also began to die in week 42 but had increased survival range to 71 weeks (p=0.03). LV-WT-HSCT delayed death to weeks 51-63 (p=0.01) with one mouse from this group still surviving at week 92. Interestingly, LV-IIIA-HSCT delayed death to weeks 57-75 (p=0.0002) with one mouse still surviving from this group at week 76. The lentiviral copy number of these LV-IIIA-HSCT mice was found to be significantly higher (0.34 copies/cell) than that of mice analysed by histology and biochemistry (0.11 copies/cell).

5.5 Discussion

This study is the first to compare a lentiviral vector based HSCT gene therapy approach against normal transplantation in Sanfilippo disease. Our data shows that although WT-HSCT is able to significantly improve many pathological markers in the brain of MPS IIIA mice it was unable to significantly improve brain enzyme activity or correct abnormal behaviour. In contrast, augmentation of brain enzyme delivery by transduction of WT HSCs with a lentiviral vector expressing the SGSH gene prior to transplant (LV-WT-HSCT), improved all pathological markers as well as, or better than WT-HSCT and normalised most of these markers to WT levels. In particular, LV-WT-HSCT was able to significantly improve brain enzyme activity to 10% of normal and fully correct behaviour of MPS IIIA mice. We also transduced MPS IIIA HSCs with a lentiviral vector expressing the SGSH gene (LV-IIIA-HSCT) and observed a phenotype somewhere between WT-HSCT and LV-WT-HSCT treatments.

HSCT in MPS IIIA patients was discontinued in the UK more than a decade ago as it was found to have no significant effect on neurological outcomes, despite some improvement in lifespan (Shapiro *et al.* 1995; Sivakumur *et al.* 1999). Our data on

WT-HSCT failure in MPS IIIA mice are consistent with these clinical observations and are also consistent with similar data obtained after whole bone marrow transplant of irradiated MPS IIIA mice (Lau *et al.* 2010a). More recently, cord blood transplantation has been performed in 19 MPS IIIA and IIIB patients (Prasad *et al.* 2008), of which 12 survived and 9 had disease stabilisation, but there was limited impact on cognitive function. In 2 patients that were transplanted before 2 years of age, modest gains in cognitive functions have been reported. Overall the transplanted patients appear to sleep better and have fewer behavioural problems, but behaviour is not corrected and this treatment remains controversial.

Our lentiviral vector is capable of effective gene expression in human microglial cells and we have previously shown it to be expressed in all murine and human hematopoietic lineages (Siapati *et al.* 2005) producing therapeutic protein for over a year in mice (Bigger *et al.* 2006). In the periphery, very significant improvements in normal enzyme activity are clearly present, but in the perfused brain only LV-WT-HSCT (10%) and LV-IIIA-HSCT (7%), were able to significantly increase enzyme activity above baseline activity of 3%, suggesting that SGSH activity from donor WT microglial cells is probably only around 3%, whilst gene therapy is providing around 4% for copy numbers of 0.11-0.27.

Total HS and HS composition were significantly altered in MPS IIIA mouse brains. We have recently shown that similar increases in amount of HS and sulphation composition, particularly in 2-*O* sulphation of HS are also present in MPS I (Holley *et al.* 2011). In the same work we also show that HS is present in significant excess in non-lysosomal and extracellular locations (Holley *et al.* 2011), and in unpublished work, show a functional role for abnormal HS sulphation in altered cell to cell signalling. Only LV-WT-HSCT was able to normalise both the amount of HS and the NAc, *N*- and 6-*O*-S sulphation patterning. Despite significant reductions in 2-*O* sulphation of HS by LV-WT-HSCT this was not normalised, suggesting that treatment could probably be improved further.

LV-IIIA-HSCT significantly reduced total HS but did not alter composition. This was the most variable treatment and this discrepancy could just reflect variable copy numbers between mice. This was particularly evident in GM2 ganglioside analysis

where some mice were corrected by LV-IIIA-HSCT and others uncorrected (Figure 5-3C). This correlated with copy number.

Both LV-WT-HSCT and WT-HSCT reduced the number of infiltrating microglial cells in the cortex by similar amounts, whilst only LV-WT-HSCT was able to correct behaviour. Microglial pathology was probably the least well corrected of all of the measured markers. This could suggest a relatively minor role for microglial infiltration in neuropathology. This is supported by work demonstrating that early neurodegeneration occurs independently of neuroinflammation in MPS IIIB mice (Ausseil *et al.* 2008). However, if microglial infiltration is a downstream event to primary pathology as seems likely, the inability to fully correct this marker could just reflect a failure of the brain to clear microglia after significant neuroinflammatory insult. Aspirin treatment of MPS IIIA mice has been shown to reduce the expression of neuroinflammatory and oxidative stress genes (Arfi *et al.* 2011), but unfortunately neither behaviour nor other markers of disease pathology such as microglial infiltration, were examined in this study, thus it is hard to know if reduction of neuroinflammation alone would significantly improve neuropathology.

It has been proposed that GM2 and GM3 gangliosides could have significant roles in neurodegeneration in MPS IIIA as they are components of lipid rafts and glycosynaptic microdomains, whilst increased GM2 gangliosides may affect signalling, adhesion, motility and growth in the brain (McGlynn *et al.* 2004). However, the ability to clear GM2 ganglioside storage whilst LAMP2 lysosomal storage and behaviour remain uncorrected, suggests that GM2 ganglioside may be less important than HS in neuropathology. It is also possible that there are other, as yet unidentified storage products that are playing a more substantial role in neuropathology. GM2 and GM3 gangliosides are stored in different vesicular compartments to HS for example (McGlynn *et al.* 2004) and HS storage is not restricted to the lysosome but is also present in the Golgi and extracellular regions (Holley *et al.* 2011). HS is probably the best indicator of pathology that we currently have but other markers such as SCMAS (Subunit c of Mitochondrial ATP Synthase) and Tau have also been proposed (Ryazantsev *et al.* 2007; Ohmi *et al.* 2009).

Neither WT-HSCT nor LV-IIIA-HSCT were able to improve behaviour overall. Although LV-WT-HSCT fully corrected behaviour, the copy number achieved was low (0.27 copies per cell) and could be improved significantly. LV-IIIA-HSCT had a variable outcome and this likely reflects the relatively variable copy number (0.08-0.55, average 0.22). This problem is most evident in the survival curves, where, against all expectation, the biggest improvement in survival was observed in the LV-IIIA-HSCT group, followed by LV-WT-HSCT and then WT-HSCT. The average copy number in the randomly selected LV-IIIA-HSCT survival cohort was 0.34, compared to 0.11 for the biochemistry cohort. This suggests that for clinical efficacy we would either require lentiviral transduction of unrelated cord blood or improved copy number in autologous transplant of MPS IIIA cells and at least 60% transduction of cells to eliminate "patchiness" in the brain. Given the improved clinical outcomes of HSCT in MPS IH with 1 year survival at 93% in our centre (Wynn *et al.* 2009a) and 91% in several European centres (Boelens *et al.* 2009), a clinical approach using unrelated CD34+ cord blood donor cells as the cell source for lentiviral transduction is potentially feasible. As we have already seen in MPS IH patients receiving HSCT from unrelated homozygote donors, there is improved metabolic correction over those receiving HSCT from related heterozygote donors (Wynn *et al.* 2009b).

The LV-HSCT approach has been applied to several other mouse models of disease. In an almost identical outcome to our own, 10% of normal *ARSA* brain expression was achieved in the mouse model of metachromatic leukodystrophy (MLD), which was associated with behavioural correction at 6 months of age (Biffi *et al.* 2006). A clinical trial has now begun using this approach. In the mouse model of MPS I, up to 450% of normal brain enzyme activity were achieved and approximately 200% was associated with brain and peripheral disease correction (Visigalli *et al.* 2010). In globoid cell leukodystrophy (GLD) approximately 16% of normal brain enzyme activity were achieved which improved survival of the mice, however comparing the mice that had less than 16% and those that had 16-25% reveals that better survival is achieved with higher enzyme activity (Gentner *et al.* 2010). It is interesting to note the difference in achievable enzyme expression in the brain in MPS I against MLD, MPS IIIA and GLD. The difference between the level of enzyme produced and the level required for correction in the brain for different enzymes

could be due to the different normal expression levels, the amount that is actually required to fulfil normal catabolism and the amount of storage. SGSH enzyme activity could be improved by co-expressing the sulphatase modifying factor 1 (*SUMF1*) gene from the vector. *SUMF1* modifies sulphatases by producing a formyl-glycine residue that is required for sulphatase activity, and over expression of *SUMF1* alongside *SGSH* increases enzymatic activity (Fraldi *et al.* 2007). There have been reports that the level of SUMF1 expression is critical to achieve significantly improved SGSH expression but codon optimisation of *SGSH* could also improve expression.

MPS IIIA still lacks any clinically approved treatment but there are three approaches in clinical development of significance. Intrathecal ERT is currently being evaluated in the clinic having shown efficacy in MPS IIIA mice (Hemsley *et al.* 2008b) and dogs (Crawley *et al.* 2011). This therapy is based on the premise that the spinal CSF can distribute drug throughout the central nervous system. It may be hampered by a requirement for repeated, long-term delivery and the normal flow of CSF which is from the brain down the spine, against the flow of any delivered enzyme. Another approach is direct injection of an AAV vector co-expressing SUMF1 and SGSH, successfully evaluated in mice (Fraldi *et al.* 2007), and now in clinical trial. It is unclear how well this treatment will scale from the 0.5cm^3 mouse brain to a 1500cm^3 human brain. Finally, we have recently shown that a substrate reduction therapy approach using high dose genistein in MPS IIIB mice was able to improve neuropathology and behaviour (Malinowska *et al.* 2010). Any of these approaches could potentially be used in combination with LV-HSCT to improve disease outcomes.

A clinical trial for LV-HSCT in MPS IIIA will need to evaluate the potential benefit of the therapy against the potential clinical risks. Retroviral mediated HSCT has been utilised in two clinical trials for X-SCID, which resulted in the successful treatment of 19 out of 20 patients using a retroviral vector. 5 patients in these two trials developed acute T cell lymphoblastic leukaemia as a result of retroviral insertional mutagenesis and one died (Hacein-Bey-Abina *et al.* 2008). However, 4 of the patients surviving leukaemia recovered T cell diversity and numbers following

chemotherapy. 30 patients have been treated for ADA deficiency worldwide using a similar approach and 67% show a clear clinical benefit with no evidence of clonal dominance (Aiuti *et al.* 2009; Gaspar *et al.* 2011b).

Since these trials, retroviral vectors have been improved by the removal of retroviral enhancer elements, which should help reduce the risk of proto-oncogene transactivation (Thornhill *et al.* 2008). Lentiviral vectors may have an improved safety profile over retroviral vectors as they tend to integrate into transcriptional units rather than promoter regions (Schroder *et al.* 2002; Wu *et al.* 2003). A recent trial for adrenoleukodystrophy using a LV-HSCT approach has shown significant delay in neurodegenerative disease progression in two patients treated so far (Cartier *et al.* 2009). Our proposed approach using LV-WT-HSCT in MPS IIIA may have a slightly increased risk from allogeneic transplant, but this may be offset by the ability to achieve neuropathological and behavioural correction using average copy numbers as low as 0.27 per cell, which will help to mitigate the risk of proto-oncogene transactivation.

In summary the LV-WT-HSCT approach significantly improved brain SGSH activity to 10% of normal activity, normalising HS storage, *N*-, 6-*O* sulphation and unsulphated HS composition, GM2 ganglioside storage and behaviour in mice with MPS IIIA. LV-WT-HSCT significantly reduces 2S HS composition and neuroinflammation with improved survival. Although both WT-HSCT and LV-IIIA-HSCT showed improvements in many of these parameters, neither treatment was able to significantly change abnormal behaviour. Given the unmet clinical need, we believe lentiviral mediated haematopoietic stem cell gene therapy is a clinically relevant and viable approach to treat MPS IIIA and similar neurodegenerative disorders.

Supplementary Video 5-1 Behavioural correction of MPS IIIA mice by LV-WT-HSCT but not other treatments

A video running at 4 times normal speed showing the median 2 minutes of video from the median mouse from the WT (top left), MPS IIIA (top right), WT-HSCT (bottom left) and LV-WT-HSCT (bottom right) groups at 6 months of age. Please see enclosed CD.

Chapter 6 – Conclusions and Future Directions

The behaviour of MPS IIIA and IIIB mice in the literature is conflicting, therefore the first aim of this thesis was to characterise the behaviour of the mouse models of MPS IIIB and MPS IIIA in order to develop tests to assess the efficacy of novel therapeutics. The second aim was to evaluate a lentiviral vector based haematopoietic stem cell gene therapy for MPS IIIA.

6.1 Comparing MPS IIIA and MPS IIIB Mouse Behaviour

When comparing the MPS IIIA and IIIB data it is important to consider the differences between these experiments. Changes were made to the open field apparatus between the MPS IIIB and MPS IIIA behavioural studies. The arena was increased in size to make it more sensitive to thigmotaxis differences (Simon *et al.* 1994; Crawley 2007), and the flooring was changed to make the video analysis of the mice easier, the different flooring could also affect behaviour, as having a sawdust flooring has been shown to facilitate running in a T maze (Contet *et al.* 2001b). The lighting was also different as the experiments were performed in a different mouse facility. We have now standardised the lighting to avoid this problem in future. Additionally the MPS IIIA mice were housed in individually ventilated cages and the MPS IIIB mice were not, any differences between housing and experimental environments can potentially affect the outcome of behavioural studies (Crabbe *et al.* 1999; Deacon 2006; Kallnik *et al.* 2007). Therefore it was not possible to make a direct comparison of the behaviour between MPS IIIA and B mice in the open field, but behavioural similarities were observed.

6.1.1 Hyperactivity

Overall hyperactivity was observed in both mouse models. The MPS IIIA mice are hyperactive at 4 and 6 months of age but at 8 months, there is a trend but they are only significantly hyperactive in the duration of motion faster than 100mm/s. Similarly the MPS IIIB mice are hyperactive but were only tested at of 8 months of age.

While the MPS IIIA and IIIB patient groups are indistinguishable there are some differences in the mice that may affect their behaviour (Heron *et al.* 2011). The MPS IIIB mice have no residual NAGLU activity while the MPS IIIA mice have approximately 3% of normal SGSH activity (Bhaumik *et al.* 1999; Li *et al.* 1999; Crawley *et al.* 2006). An ongoing study in this laboratory by Ai Yin Liao is currently analysing the behaviour of the MPS IIIB mice at 6 months of age in the same open field that we used for the MPS IIIA behaviour, so we will be able to make a direct comparison in the future. It would be interesting to compare the activity of the MPS IIIA and MPS IIIB mice in the same open field set up at different time points as the low level of residual enzyme activity in MPS IIIA mice may cause a delayed onset or a less pronounced hyperactive phenotype. The sense of danger in the open field may also be affected. However, work by (Hemsley *et al.* 2005) suggests that the behaviour phenotype could be present from 3 weeks of age in MPS IIIA, although the behaviour of the MPS IIIA mice is conflicting in the literature.

Further work could be performed on the timing of the one hour open field. Is it important that it is performed during a peak of activity identified in the circadian rhythm? Or is the consistency in the circadian time point important (Kopp 2001)? Previous studies that utilised a one hour open field test did not observe hyperactivity but they were performed at different points in the circadian rhythm and were also measured differently (Fu *et al.* 2007). If the circadian time point has an effect this may have an impact on the use of open field tests in the wider behavioural research field. However, previously it has been shown that social behaviours are not affected by testing in the light or the dark phase (Yang *et al.* 2008).

6.1.2 Reduced Sense of Danger?

The MPS IIIA mice demonstrated a reduced sense of danger at 4 and 6 months but not at 8 months as measured by the centre duration in the open field. This is consistent with the MPS IIIB mice which were examined at 8 months of age and no difference was observed. However a small open field was used with the MPS IIIB mice which may be less sensitive to sense of danger (thigmotaxis) differences

(Crawley 2007). No difference was also observed by Lau *et al.* with MPS IIIA mice that also used a small open field (Lau *et al.* 2008).

The elevated plus maze was also used to assess the sense of danger in MPS IIIA mice but no differences were observed at 4, 6 and 8 months of age. The elevated plus maze has previously yielded conflicting results in MPS IIIB mice, a reduced sense of danger (increased entry to open arms) was observed when the test was performed in the dark (Cressant *et al.* 2004) but not in the light (Fu *et al.* 2007). In the MPS IIIA mice a reduced sense of danger has been observed at some time points but not others (Lau *et al.* 2008) and in some papers no difference is observed (Lau *et al.* 2010a).

The discrepancy in the reduced sense of danger in the open field but not in the elevated plus maze could have been because the elevated plus maze was performed 30 minutes after the open field and previous behavioural tests can affect the results of subsequent tests (Voikar *et al.* 2004). The width of the elevated plus maze arms in this study are wider than in some other studies (Lau *et al.* 2008), which has been shown to have an effect on some strains of mice but not others (Lamberty *et al.* 1996). However narrower arms can make it harder to place the mice consistently into the maze and to clean it adequately (Deacon *et al.* 2006).

Therefore to clarify if MPS IIIA and IIIB mice demonstrate a reduced sense of danger or reduced anxiety, the elevated plus maze should be repeated on näively tested mice. If we do not observe a reduced sense of danger in the test the elevated zero maze, elevated platform or 3D spatial maze could be tested (Crawley 2007; Michalikova *et al.* 2010). Alternatively the mice may have a reduced sense of danger in the open field because they have reduced thigmotaxis, but they have a normal or heightened response to the transition from the dark enclosed arms to the brightly lit open arms, this could be evaluated in a light-dark exploration test (Crawley *et al.* 1980). This may explain why increased MPS IIIB entries into the open arms of the elevated plus maze have been observed in the dark (Cressant *et al.* 2004) but not in the light (Fu *et al.* 2007).

6.1.3 Neuromuscular Strength

In MPS IIIB mice the inverted screen and horizontal bar crossing tests that measure neuromuscular strength and motor coordination, showed no significant differences at 8 months of age and a significant (horizontal bar crossing) or close to significant (inverted screen) decline in MPS IIIB mice at 10 months of age. This suggests that the neuromuscular strength tests only have utility after 8 months of age. The decline in motor function coincides with urine retention in the mice which may impact on their ability to perform these tests (Bhaumik *et al.* 1999; Gografe *et al.* 2009; Malinowska *et al.* 2010). The lack of neuromuscular changes in MPS IIIB mice suggests that the open field behaviour observed is due to neurological aspects of disease and not somatic aspects. Likewise in MPS IIIA at 4, 6 and 8 months of age no neuromuscular strength or coordination differences in male or female mice were detected. In MPS IIIB mice there was a trend towards reduced neuromuscular strength and coordination but not at 8 months with the MPS IIIA mice, this could be because of the residual enzyme activity in the MPS IIIA mice delaying onset of disease. Therefore the neuromuscular behaviour of the MPS IIIA mice at later time points could be examined to determine if a delay is observed but it would not be of practical use in testing novel therapeutics unless long-term experiments were performed.

A decline in motor function after 8 months of age in MPS III is supported by most of the literature (Crawley *et al.* 2006; Fu *et al.* 2007; Heldermon *et al.* 2007; Fu *et al.* 2010; Heldermon *et al.* 2010) except (Hemsley *et al.* 2005).

A factor that needs to be addressed is the question of what a behavioural assay is actually measuring. A recent paper using MPS IIIB mice suggested that it had demonstrated “correction of cognitive and motor function” using an accelerating Rotarod test at 5-5.5 months of age (Fu *et al.* 2011). This test has previously shown no differences at this or later ages and it seems a stretch to say that is measuring cognitive function as most consider it measures neuromuscular strength and coordination. Therefore it is advantageous to have correction of a range of complex behaviours such as hyperactivity and anxiety rather than just motor function. It would also be interesting to correct the somatic disease and then examine the

behaviour of the mice to confirm that the behavioural test is measuring effects on neurological function. Peripheral correction could be achieved with intravenous delivery of lentiviral vector or hydrodynamic delivery of plasmid to produce high activity of enzyme that is unlikely to cross the blood brain barrier.

6.1.4 Memory and Learning

We have not performed any analysis of learning and memory in the MPS IIIA or IIIB mice as these tests are very time consuming to perform so are not ideal for testing a large number of mice to assess therapeutic effect. The MPS IIIA and IIIB mice are reported to have a reduced spatial learning ability. For MPS IIIB, a Morris water maze (Fu *et al.* 2007) and a hidden Morris water maze (platform is not visible) (Fu *et al.* 2010) have been performed. In the standard Morris water maze over three days 4.5-5 month old WT mice improve in finding the platform from about 22s to about 4s, the MPS IIIB mice perform significantly worse but do improve from about 39 seconds to about 19 seconds (Fu *et al.* 2007). However the WT mice swim at 17 cm/s but the MPS IIIB mice only swim at around 9 cm/s, therefore the MPS IIIB mice may have a reduced memory but their reduced swim speed is confounding the analysis; it might take them longer to get there because swimming is more difficult.

A hidden platform Morris water maze was performed at 5-5.5 months of age, this controls for different visual acuity between WT and MPS IIIB mice, there was a much smaller decrease in time to platform by WT mice, (~35s to ~28s) but it was significantly different from MPS IIIB (~44s to 42s) (Fu *et al.* 2010). With this test there was still a significantly slower swim speed in the MPS IIIB mice but the difference was not as great (0.97cm/s for WT and 0.83cm/s for MPS IIIB) and the speed was much slower than previously reported (17cm/s and 9cm/s). Therefore in the hidden water maze, where the mouse cannot see the platform, there is less difference between WT and MPS IIIB mice but the test is still affected by differences in swim speed.

In MPS IIIA, a different protocol was performed for the hidden Morris water maze. In a 5 day acquisition phase, after the first day, the WT mice reached the platform faster on each day in one study (Gliddon *et al.* 2004) but only on one day in another (Crawley *et al.* 2006). After the acquisition phase the platform was removed and

the time spent in the correct quadrant was measured. A significant difference was detected between WT and MPS IIIA in both papers but in one, the difference quoted in the text could not have been significantly different and did not match the figures (Crawley *et al.* 2006). A relearning phase was then carried out after the probe phase and in both papers MPS IIIA mice performed significantly worse. These papers did not report any differences in swim speed. Another study in MPS IIIA was performed slightly differently. For two days before the acquisition phase the mice could see the platform and no difference between WT and MPS IIIA was observed. In the 5 day acquisition phase MPS IIIA mice were slower in only two days (Fraldi *et al.* 2007). In the probe phase the difference between WT and MPS IIIA was not statistically analysed but there was a definite trend towards a reduction in time spent in the correct quadrant by MPS IIIA mice. This paper did not present the swim speeds but reported that they were not significantly different.

It appears that MPS IIIA and IIIB mice have a reduction in spatial learning and memory but differences in visual acuity or swim ability may confound the results. Consequently it needs to be determined whether MPS IIIA and B mice have a reduced swimming ability, if they do then the Morris water maze should not be used (Crawley 2007). The Morris water maze could still be used if the distance travelled in getting to the platform was analysed rather than the time but using alternative memory tests would be better (Contet *et al.* 2001a).

An alternative to the Morris water maze is the Barnes maze (Barnes 1979). In this test the mouse is placed in the centre of a brightly lit table with holes around the edge and one of these holes is an escape to a dark compartment. However the hyperactivity and possible reduced sense of danger in the MPS IIIA and IIIB mice may affect this learning test. Therefore a better test for learning and memory in the MPS mice may be the radial maze or T maze, where the order of arm entries and the arms entered are recorded (Deacon *et al.* 2006). WT mice tend to explore each area and remember where they have explored; therefore the amount of time the mouse takes is not relevant so the hyperactive behaviour or reduced sense of danger should not confound the results.

6.1.5 Optimisation of Behavioural Testing

The open field analysis in MPS IIIA was improved by using a larger open field, performing the test for longer (60 minutes compared to 3 minutes) at the same point of circadian rhythm and by using video analysis software which provides better spatial and temporal resolution (Kafkafi *et al.* 2003; Benjamini *et al.* 2010). This should make this open field set up more reliable than previously used in the literature and offer a more sensitive assessment of therapeutic effect. Previous studies in MPS IIIA only measured the behaviour in the open field in the first three minutes, in our hands the first three minutes was not indicative of the behaviour over 60 minutes. Consequently some of the variability in the literature could be caused by different operators putting the mice in the open field in different ways, over only 3 minutes this could have a large impact on the results.

At the start of the study it was not known which sex would produce consistent behaviour outcomes and if transplant alone would affect behaviour, therefore 10 groups of mice with 10 per group were transplanted. In addition untreated control groups (WT and MPS IIIA, male and female n=10) were also tested. All mice were put through all the behavioural tests at 4, 6 and 8 months of age. The behaviour of WT mice that received a transplant of WT cells was not different to un-transplanted WT controls and therefore was not included in the results. The male mice showed no behaviour differences, although they almost significantly spent more time in the centre of the open field at 6 months ($p=0.055$). Hence we did not analyse these mice in the long term study of lentiviral enhanced stem cell gene therapy and only analysed the female mice. The differences in behaviour between sex could be due to the fact that the male mice were singly housed due to aggressive behaviour while the females were group housed (Voikar *et al.* 2005; Arndt *et al.* 2009).

In the future, to evaluate novel therapies in MPS IIIA we will only use female mice as they display the behavioural changes and are more economical to house. Additionally WT to WT transplants or the WT to MPS IIIA transplants will not need to be performed. The behaviour will only need to be assessed at 6 months of age and rather than perform the open field test, repeated elevated plus maze, bar crossing and inverted screen test, only the one hour open field test will be

performed. This will speed up and simplify the assessment of therapies in the MPS IIIA mice in our lab.

6.1.6 MPS III Mouse and Patient Behavioural Similarities

The MPS III patient's behavioural problems include hyperactivity, a reduced sense of danger, aggression and sleep disturbances (Cleary *et al.* 1993; Bax *et al.* 1995; Fraser *et al.* 2002; Fraser *et al.* 2005; Valstar *et al.* 2008; Malm *et al.* 2010). The results of these mouse behaviour papers concur with the patient phenotype. Hyperactivity is observed in MPS IIIA and IIIB mice and, with a larger open field, a reduced sense of danger is observed in MPS IIIA mice. Aggression in male MPS IIIA mice has been observed and resulted in singly housing the male mice but this has not been quantified (Gliddon *et al.* 2004). Changes in the circadian rhythm have also been observed in MPS IIIB mice (Canal *et al.* 2010). Therefore in terms of hyperactivity, a reduced sense of danger, aggression and sleep disturbances the MPS III mouse models appear to be a good model of patient disease.

Professor Elsa Shapiro is undertaking a natural history study of MPS IIIA patients funded by Shire Human Genetic Therapies, Inc. (NCT01047306). At the MPS III Research & Treatment Workshop in 2011 she presented preliminary data speculating that MPS IIIA has similarities with Klüver–Bucy syndrome where loss or damage of the amygdala results in behavioural changes. Klüver–Bucy syndrome was first identified by making large resections in the temporal lobes of rhesus monkeys (Klüver *et al.* 1937). Patients have visual agnosia (the inability to recognise familiar objects or faces), hyperorality (an urge to examine objects with the mouth), hypermetamorphosis (the impulse to notice and react to everything within sight), docility, loss of the normal fear or anger response, hypersexuality and dietary changes. Also reported is aphasia (impairment of language), memory disturbance, seizures, aggression, uncontrolled screaming and hyperactivity (Cohen *et al.* 2010), although patients may not exhibit all of the symptoms listed. Some of these behaviours, such as the reduced fear, aggression and hyperactivity have been observed in the MPS III patients and mice but many including hypersexuality have not been reported (Cleary *et al.* 1993; Bax *et al.* 1995; Fraser *et al.* 2002; Fraser *et al.* 2005; Valstar *et al.* 2008; Malm *et al.* 2010; Wegrzyn *et al.* 2010).

The amygdala is involved in emotional responses and so can be measured by a contextual or cued fear experiment (Phillips *et al.* 1992). A mouse is given a mild shock in a novel environment after a noise has been made. The next day the mouse is reintroduced to the environment without the sound and the freezing behaviour is measured, this is contextually conditioned fear. The next day the mouse is placed in a new environment and the noise is made and the freezing behaviour is recorded, this is cued or tonal conditioning (Crawley 2007). This test has been performed on 2.5 and 4.5 month old MPS IIIB mice; no difference in contextual fear was observed and no difference in tone fear at 2.5 months but a reduced tone fear response was observed at 4.5 months. However the lack of tonal fear at 4.5 months could be due to hearing deficits observed in MPS IIIB mice (Li *et al.* 1999). Therefore the MPS IIIB mice do not present with contextual fear differences that you would expect from amygdala damage in Klüver–Bucy syndrome.

To further examine the behavioural similarities to Klüver–Bucy syndrome the aggression of MPS IIIA and B mice could be quantified using the resident intruder test (Thurmond 1975). Alongside this the social interactions of the mice could be examined with novel mice or cage mates.

The amygdala may play a role in the MPS III behavioural phenotype and we and others have observed accumulation of GM2 in this area (McGlynn *et al.* 2004; Lau *et al.* 2010a) but GM2 storage is also present throughout the brain and the patients and mice do not appear to present with all behavioural aspects. Therefore as there is global accumulation of HS, extensive neuroinflammation and widespread accumulation of GM2 gangliosides it is possibly too restrictive to consider the amygdala storage of GM2 gangliosides as the cause of the behaviour phenotype. Any theories of the cause of the behaviour of MPS III patients will need to explain the differences in behaviour between MPS I and III patients (Wegrzyn *et al.* 2010). While MPS III patients are reported to be hyperactive, aggressive and have a reduced sense of danger, MPS I patients have little behavioural problems other than learning difficulties despite both storing HS (Pitt *et al.* 2009; Wegrzyn *et al.* 2010).

In preliminary data from Dr Michael Potegal, MPS I and MPS III patients spent time in a "risk-room" with their mother and their exploratory behaviour and behaviour when their mother left was examined. The MPS I patients explored less than the MPS III patients and the MPS III patients were not as concerned by the absence of their mother (Potegal *et al.* 2011). These behavioural differences are despite both MPS I and MPS III storing HS. Therefore the behaviour of the mouse models of MPS I and III could be examined further to try and corroborate this observation however, the more complex behaviours observed in the patients may not be quantifiable in mice. However it is hard to compare behaviour of MPS I and MPS III mice as the MPS I mice have more severe somatic and skeletal manifestations.

6.1.7 MPS III Mouse vs. Patient Disease Pathology and Response to Treatment

In our laboratory Dr Fiona Wilkinson has recently examined the neuropathology of MPS I, IIIA and IIIB mice at 4 and 9 months of age (Wilkinson, Submitted PLoS ONE September 2011). An increase in the size of the lysosomal compartment in MPS IIIB compared to MPS I and increasing storage from 4 to 9 months was observed. GM2 ganglioside storage was observed throughout the brain but more intense storage was observed in several brain regions including the amygdala in both MPS I and III. The storage of GM2 ganglioside in the cerebral cortex was quantified and there was no significant difference between MPS types or from 4 to 9 months of age. Neuroinflammation in terms of activated astrocytes (GFAP positive) and microglia (isolectin B4 positive) was significantly increased in MPS IIIA and B in comparison to MPS I but this parameter also does not progress from 4 to 9 months of age. No significant neuronal cell loss or reduction in cortical thickness was detected but a trend towards a reduction was observed. The combination of no changes in GM2 gangliosides and neuroinflammation from 4-9 months suggests these factors are less important in neurodegeneration and behavioural phenotype, and primary storage that increases with time is more important. Previously neurodegeneration has been shown to occur independently to neuroinflammation in MPS IIIB mice and that anti-inflammatory administration to MPS IIIA mice had no effect of the expression of apoptosis genes (Ausseil *et al.* 2008; Arfi *et al.* 2011).

The study of lentiviral vector stem cell gene therapy in chapter 5 suggests that GM2 gangliosides and neuroinflammation are not important in determining the behaviour of MPS IIIA mice as the LV-WT-HSCT and WT-HSCT treatments had the same effect on GM2 gangliosides and neuroinflammation in the cortex but LV-WT-HSCT corrected the behaviour and WT-HSCT did not (Table 6-1). There appeared to be little or no effect on GM2 ganglioside storage in the amygdala by both treatments (Figure 5-4). The difference between these treatment groups was that there was less HS storage in LV-WT-HSCT and the HS was less sulphated. The amount of HS and the sulphation state of HS is important in many cellular process including cell signalling and growth factor signalling and gradients (Merry *et al.* 2002; Bishop *et al.* 2007; Turnbull *et al.* 2010). In the work of Dr Wilkinson in our lab it was also found that the HS in MPS IIIA and IIIB is more sulphated than in MPS I or WT. The difference between WT-HSCT and LV-WT-HSCT could be further analysed to examine the differences in GM2 and GM3 gangliosides by thin layer chromatography but we have currently been unable to do so. Examination of VAMP2 and synaptophysin, proteins involved in neuronal synapses or cholesterol that is also found to be elevated in MPS could also be examined in different regions of the brain, including the amygdala.

Brain	WT	LV-WT-HSCT	LV-IIIA-HSCT	WT-HSCT	MPS IIIA
SGSH Activity, % WT	100	10	7	6	3
Total GAGs, Fold WT	1	1.1	0.7	1.2	1.6
Total HS, Fold WT	1	5	9	9	17
%HS Un sulphated	58	52	39	48	38
%HS NS	42	48	61	52	62
% HS 6S	21	22	29	23	31
% HS 2S	20	32	48	38	52
Cortex Microglia Number	8	62	76	61	143
Cortex GM2 gangliosides, Fold WT	1	28	99	25	110
Hyperactive Behaviour (Path length, m) 4 months	110	119	155	186	195
Hyperactive Behaviour (Path length, m) 6 months	77	79	151	118	181
Sense of danger (Centre entries) 4 months	168	204	248	329	300
Sense of danger (Centre entries) 6 months	99	107	250	164	273

Table 6-1 Comparison of treatment results from chapter 5
A summary of the biochemistry and histology results in the brain and behavioural outcomes. The path length and centre entries are indicative of the other activity and sense of danger measures respectively. This table highlights the different behavioural outcomes in comparison to biochemical and histology results.

MPS IIIB is also thought to be a tauopathy like Alzheimer's disease; hyperphosphorylated tau was detected in the medial entorhinal cortex and in older mice in the dentate gyrus, which are regions involved in memory and memory and navigation respectively, but the mechanism of storage is not clear (Ohmi *et al.* 2009). However it is not clear if hyperphosphorylated tau in this very limited region of the brain involved in memory is relevant to the clinical pathology or treatment of MPS III. Additionally some of the antibodies against hyperphosphorylated tau used in Alzheimer's disease did not work in MPS IIIB (Ohmi *et al.* 2009) and two prior studies did not observe hyperphosphorylated tau (Ginsberg *et al.* 1999; Hamano *et al.* 2008). Therefore it seems unlikely that the very limited distribution of hyperphosphorylated tau that one group has observed is the most important factor in MPS III. The brains of the treated and untreated mice in this study could be examined to see if the treatment has had any effect on tau expression.

We propose that the behaviour of MPS III is most likely to be related to the amount and sulphation of HS rather than the presence of GM2 gangliosides, neuroinflammation and hyperphosphorylated tau in specific regions of the brain but more work is needed to confirm this hypothesis.

6.1.8 Impact of Behavioural Papers

The results of these behavioural studies probably does not have broad applicability to other MPS diseases or LSDs, as MPS III is unusual in having a primarily neuronal phenotype with mild somatic effects. Mice with significant skeletal pathology may have impaired movement and so in the open field test hypoactivity may be observed where hyperactivity is expected. Therefore this test would not measure correction of neurological behaviour but effect on the body.

The behaviour of MPS I and MPS VII mice has been described in the literature but there are inconsistencies. MPS I mice (Clarke *et al.* 1997) are either hypoactive (Pan *et al.* 2008) or show no difference in path length (Reolon *et al.* 2006), and no difference in centre time (Reolon *et al.* 2006; Pan *et al.* 2008) in the open field test. No differences in anxiety were observed in the elevated zero maze or marble burying tests (Pan *et al.* 2008). Some groups have observed no difference in spatial memory in the Morris water maze (Pan *et al.* 2008) but others show a decrease

(Wolf *et al.* 2011). Other memory tests such as novel object recognition have been used which show that MPS I mice spend more time examining a novel object when it is reintroduced at 8 months of age (Pan *et al.* 2008) but not younger (Reolon *et al.* 2006; Pan *et al.* 2008). Further memory tests have been performed which show no difference in short term inhibitory avoidance but a reduction in long term inhibitory avoidance (Reolon *et al.* 2006).

Since there is no clear behavioural phenotype in MPS I patients or the mice, 5 minute open field tests were performed 3 times separated by 30 minute gaps. The WT mice were 55% less active in the third than first trial but the MPS I mice were only 31% less active (Pan *et al.* 2008). This suggests that the MPS I mice habituate to a novel environment more slowly or are hyperactive compared to WT when measured in this way but not in a long open field test (up to 90 minutes has been analysed) where the somatic manifestations may be more limiting. This repeat open field test is frequently used in MPS I mice (Hartung *et al.* 2004; Pan *et al.* 2008; Wang *et al.* 2009; Visigalli *et al.* 2010) but has not been tested in MPS III. By using a repeat test the effect of any neuromuscular affects on behaviour can be controlled although it has not been determined if neuromuscular strength and coordination defects are present in MPS I mice.

MPS VII mice have not been examined by the open field test but they are hypoactive in home cage activity and have circadian rhythm changes (Ross *et al.* 2000). They also spend less time grooming, which is thought to be caused by skeletal problems (Chang *et al.* 1993; Bastedo *et al.* 1994). MPS VII mice have a reduced swimming ability (Chang *et al.* 1993) that could affect the reduced spatial memory in MPS VII mice in the Morris water maze (Chang *et al.* 1993; Bastedo *et al.* 1994; O'Connor *et al.* 1998; Sakurai *et al.* 2004). However the MPS VII mice also have impaired novel object recognition (Fukuhara *et al.* 2006) and in a repeated acquisition and performance chamber appear to have memory problems (Brooks *et al.* 2002).

The behaviour of MPS II mouse models has not been evaluated (Garcia *et al.* 2007; Jung *et al.* 2010) and the behaviour papers from this study may influence the characterisation of these mice. The behavioural measures identified in this study

will be used to characterise the MPS IIIC mouse model that has recently been made in Dr Alexei Pshezhetsky's laboratory.

6.1.9 Novel Mouse Model Behavioural Testing Strategy

Assessing the behaviour of MPS IIIA and IIIB mice and reviewing the literature has led me to devise a plan for testing a novel MPS mouse model. Firstly the neuromuscular strength and coordination of the mice should be analysed as subsequent tests can be affected by neuromuscular problems. A simple way to do this is to use the bar crossing and inverted screen test (Jeyakumar *et al.* 1999) or use an accelerative or rocking Rotarod. The open field or repeated open field test (if the mice have neuromuscular problems) is also a simple test that is easy to perform that can be used to examine the activity and habituation of the mice and also indicates if the mice have an altered sense of danger (or anxiety) by the amount of time spent in the centre. The time of day the open field test is performed could also be important so a study of the circadian rhythm activity may also be beneficial. If the open field test suggests that there are anxiety differences this can be confirmed with elevated plus or zero mazes. If the researcher has observed neuromuscular strength differences or hypo or hyper activity differences they should consider how these may impact on the analysis of these mazes. It may be most appropriate to examine the number of entries into the open as a percentage of all arm entries, but multiple parameters should be considered (distance, time, entries, percentage of distance, percentage of entries) to make an informed assessment of the results.

Tests of cognitive function and memory are very time consuming to perform so are not ideal for testing novel therapeutics; however they are useful in characterising a mouse model. The most commonly used spatial memory test is the Morris water maze, however the swimming ability must be assessed and the eyesight of the mice should be tested if visual cues are going to be used. If the swimming ability is significantly reduced the distance travelled to the platform or the proportion of the path length in the correct quadrant could be measured using video tracking software, however it may be more appropriate to perform alternative memory tests such as the barns, radial or T maze. The radial or T maze may be most applicable if there are activity level differences. Other memory tests such as the

contextual or tonal fear tests can also be performed, but the visual and auditory acuity should be examined to confirm that the results are caused by memory defects not an inability to recognise the surroundings or hear the sound.

Aggression and social behaviours could also be examined using resident intruder tests and other behaviour test dependent on the patient phenotype and any anomalies in the other tests.

6.2 Lentiviral Vector Enhanced Haematopoietic Stem Cell Gene Therapy for MPS IIIA

Having optimised the analysis of behaviour in MPS IIIA mice the effect of lentiviral vector based haematopoietic stem cell gene therapy for MPS IIIA in the mouse model was assessed. There are two potential approaches to clinically treat MPS IIIA patients with lentiviral vector based haematopoietic stem cell gene therapy. Firstly donor bone marrow or cord blood could be transduced with the lentiviral vector, which we have modelled as the LV-WT-HSCT, or an autologous transplant where the patient's own bone marrow is gene modified before being returned to the patient; modelled using MPS IIIA bone marrow (LV-IIIA-HSCT). These approaches have been compared to a donor transplant with cells expressing normal SGSH activity (WT-HSCT), which has been shown to be ineffective in improving neurological function in patients although it may stabilise the peripheral disease (Vellodi *et al.* 1992; Shapiro *et al.* 1995; Sivakumur *et al.* 1999; Lau *et al.* 2010a).

For evaluation of the lentiviral enhanced stem cell gene therapy in the MPS IIIA mice a number of experiments had to be optimised: the transplantation of MPS IIIA mice with low numbers of lineage depleted haematopoietic stem cells, the production and titration of the SGSH lentiviral vector. Titration of the vector involved setting up QPCR and designing and testing different primer and probe sets and producing a known copy number standard. The SGSH enzyme activity assay was optimised further and the MPS IIIA mice were backcrossed onto the C57BL/6 background so that the behavioural testing and analysis could be optimised and performed.

6.2.1 The LV-IIIA-HSCT or LV-WT-HSCT Approach in the Clinic

The LV-WT-HSCT transplant was the most successful but with higher copy numbers or enhanced expression of SGSH the LV-IIIA approach would be indistinguishable from the LV-WT approach. The LV-WT approach has the advantage that lower copy numbers would be required because of the endogenous expression of SGSH which would make it safer from an insertional mutagenesis stand point. However the LV-IIIA approach would involve a safer conditioning regimen with less immune suppression required and no risk of graft vs. host disease. It should also be considered that despite the risks there are currently no treatments for MPS IIIA and patients die on average at 15 years of age (Meyer *et al.* 2007; Heron *et al.* 2011).

In some diseases such as X-SCID, the transplanted transduced autologous cells have a selective advantage over the resident HSCs therefore little or no conditioning is required (Qasim *et al.* 2009). However in MPS IIIA where there is little or no selective pressure, myeloablative conditioning may still be required. Immune suppression may also be necessary to prevent a response against the transgene (Cartier *et al.* 2009; Grez *et al.* 2011). Therefore even in an autologous transplant, a high level of myeloablation and immune suppression may be required. In ongoing work in our laboratory Kia Langford-Smith has been working on reduced intensity transplant conditioning regimens in WT and MPS I mice that may also be applicable in MPS III.

This study cannot advise which approach is most appropriate for the clinic but it does strongly suggest that the lentiviral vector haematopoietic stem cell gene therapy approach is an efficacious treatment for MPS IIIA and potentially other neuropathic lysosomal storage disorders with secreted enzymes.

Additionally, this study also cannot advise on the best time to treat patients and whether MPS IIIA disease is reversible. Although MPS IIIA mice at 8 weeks of age are considered symptomatic (Crawley *et al.* 2006) there is no significant neuronal loss in the mice at 9 months of age although there is a trend (Dr Fiona Wilkinson unpublished data). In advanced patients LV-HSCT may just stabilise the disease like a normal transplant and not improve the quality of life. The younger MPS I patients are transplanted the better the outcome and this is likely to be the case for MPS III

(Boelens *et al.* 2007; Boelens *et al.* 2009). There is no newborn screening program for MPS IIIA and given the rarity of the condition there is unlikely to be one. However pre-symptomatic patients are identified where a sibling or other family member has already been diagnosed.

6.2.2 Clinical Development of Lentiviral HSCT

The lentiviral vector backbone used in this study would not gain regulatory approval from the gene therapy advisory committee (GTAC) since it contains a viral promoter. The vector backbone has now been modified in this lab by Ana Sergijenko who has changed the backbone to one that is currently in use in the clinical trial in Italy for MLD by Dr A. Biffi and Professor L. Naldini. This vector uses a human PGK or human CD11b promoter for the SGSH gene rather than the viral SFFV promoter; this reduces the chance that the promoter will cause transcription to start at neighbouring genes to the integration site. The wpre sequence has also been mutated to stop the production of the X protein that has been implicated in oncogenesis (Schambach *et al.* 2006). The *SGSH* gene sequence has now been codon optimised to enhance expression of SGSH, and the length of the transgene has been shortened by removing the untranslated region to enhance transduction. Co-expressing the sulphatase modifying factor 1 (*SUMF1*) gene which modifies sulphatases by producing a formyl-glycine residue that is required for sulphatase activity could also increase SGSH activity (Fraldi *et al.* 2007).

The safety and efficacy of this vector is being examined in the laboratory by Ana Sergijenko *in vitro* in haematopoietic stem cells and *in vivo*. The Modlich assay will be performed to look at the rate of oncogenic transformation with the lentiviral vector *in vitro* (Modlich *et al.* 2006). This is a requirement for regulatory approval from GTAC and the Medicines and Healthcare products Regulatory Agency (MHRA). *In vivo* efficacy of this therapy will be assessed in MPS IIIA mice to establish if higher copy numbers and higher activity of SGSH are achieved in the brain with a greater reduction in HS. The incidence of leukaemia *in vivo* will also be examined. Integration site analysis will be performed to determine if there are common integration sites, or common integrations into genes within common integration sites (Biffi *et al.* 2011).

6.2.3 Other Therapies in Development

There are two main alternative therapeutic approaches that are currently in or starting soon in clinical trial, intrathecal enzyme replacement therapy (IT-ERT) or intracranial AAV gene therapy.

IT-ERT is currently in clinical trial in Manchester by Shire Human Genetic Therapies Ltd for MPS IIIA (NCT01299727, NCT01155778). Recombinant human SGSH is being delivered by intrathecal drug delivery ports monthly in the first cohort but rising to fortnightly in another. However there is a risk that the port can become infected (Boviatsis *et al.* 2004) and the behavioural problems of MPS IIIA children makes it hard to get them to cooperate for the injections. This therapy will require lifelong repeated injections if it is successful. Preclinical work in MPS IIIA mice (Savas *et al.* 2004; Hemsley *et al.* 2007; Hemsley *et al.* 2008a) and dogs (Hemsley *et al.* 2009b; Crawley *et al.* 2011) is promising but higher doses are required for therapeutic effect in the dog than are being used in the clinical trial. This may also be a very expensive way to treat patients as the production of recombinant enzyme is expensive and repeat delivery for life will be necessary.

Lysogene announced in June 2011 that authorization for a phase I/II clinical trial (SAF-301) of direct injection of AAV had been approved by the French Agency for the Safety of Health Products (AFSSAPS) and that the clinical trial began in September 2011. This approach has varying results in preclinical testing with apparent success in some areas but is limited by the spread of AAV so that no overall reduction in HS was observed (Fraldi *et al.* 2007). However in short term preclinical testing of intrastriatal delivery of the GMP clinical vector in MPS IIIA mice performed in this laboratory for Lysogene, we have seen higher activity of SGSH expression although this decreases further from the injection site. Direct injection of AAV into MPS IIIB mice was more successful (Fu *et al.* 2002; Fu *et al.* 2007; Heldermon *et al.* 2010). It is currently unclear how well this treatment will scale from the 0.5cm^3 mouse brain to a 1500cm^3 human brain and how many injection sites will be required. However a clinical trial for late infantile neuronal ceroid lipofuscinosis (NCT00151216) has shown some success in reducing the rate of decline but, an antibody response was raised against the vector capsid and it is not

clear if the adverse events that occurred in the trial were caused by the disease, the vector or surgery (Worgall *et al.* 2008). A new clinical trial is now underway with an AAV serotype that has a better distribution and expression in the brain (NCT01161576).

In both these approaches the somatic manifestation, although mild, will also require treatment through gene therapy or enzyme replacement therapy. There is also the concern in both of these treatments that an immune response will limit the efficacy of the enzyme or cause neuroinflammation. An immune response against the enzyme will not occur in LV-HSCT as the immune system is replaced with one that expresses SGSH, therefore the immune system will be tolerant to SGSH.

We have recently shown that a substrate reduction therapy approach using high dose genistein in MPS IIIB mice was able to improve neuropathology and behaviour (Malinowska *et al.* 2009; Malinowska *et al.* 2010) and a clinical trial in MPS III is being planned. Any of these approaches could ideally be used in combination with LV-HSCT to provide a source of the deficient enzyme to improve disease outcomes.

6.3 Conclusions

This thesis set out to firstly to evaluate the behaviour of MPS IIIA and IIIB mice and relate this to the patient phenotype and to determine a behavioural test and the appropriate age to evaluate novel therapies in MPS IIIA.

The behavioural testing has allowed us to establish that both MPS IIIA and IIIB mice share behavioural similarities with the MPS III patients; they are hyperactive and at least in the MPS IIIA mice exhibit a reduced sense of danger in the open field. These two supporting papers have allowed us to identify a robust assay with multiple parameters to evaluate the effect of lentiviral vector enhanced haematopoietic stem cell gene therapy on the neurodegeneration in MPS IIIA mice.

The second aim was to over express SGSH in haematopoietic stem cells using a lentiviral vector and transplant these cells into MPS IIIA mice. The third aim was to perform a long-term comparison of lentiviral vectors expressing SGSH in

transplanted MPS IIIA HSCs compared to transplants of transduced normal cells and normal cells alone into MPS IIIA mice, to determine the effect on enzyme activity, GAG and HS storage levels, HS sulphation, GM2 ganglioside secondary storage, neuroinflammation and behaviour.

This thesis has demonstrated that the lentiviral enhanced haematopoietic stem cell gene therapy approach to treat a mouse model of MPS IIIA is viable, and warrants further in vitro investigation before being taken forward into clinical trial.

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Appendix

Hyperactive Behaviour in the Mouse Model of Mucopolysaccharidosis IIIB in the Open Field and Home Cage Environments

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Hyperactive behaviour in the mouse model of mucopolysaccharidosis IIIB in the open field and home cage environments

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Mucopolysaccharidosis IIIB (MPS IIIB) is a lysosomal storage disorder characterized by severe behavioural disturbances and progressive loss of cognitive and motor function. There is no effective treatment, but behavioural testing is a valuable tool to assess neurodegeneration and the effect of novel therapies in mouse models of disease. Several groups have evaluated behaviour in this model, but the data are inconsistent, often conflicting with patient natural history. We hypothesize that this discrepancy could be due to differences in open field habituation and home cage behaviour. Eight-month-old wild-type and MPS IIIB mice were tested in a 1-h open field test, performed 1.5 h after lights on, and a 24-h home cage behaviour test performed after 24 h of acclimatization. In the 1-h test, MPS IIIB mice were hyperactive, with increased rapid exploratory behaviour and reduced immobility time. No differences in anxiety were seen. Over the course of the test, differences became more pronounced with maximal effects at 1 h. The 24-hour home cage test was less reliable. There was evidence of increased hyperactivity in MPS IIIB mice, however, immobility was also increased, suggesting a level of inconsistency in this test. Performance of open field analysis within 1–2 h after lights on is probably critical to achieving maximal success as MPS IIIB mice have a peak in activity around this time. The open field test effectively identifies hyperactive behaviour in MPS IIIB mice and is a significant tool for evaluating effects of therapy on neurodegeneration.

Keywords: Behaviour, lysosomal storage disease, MPS IIIB, mucopolysaccharidosis, neurodegenerative disease, open field, Sanfilippo syndrome

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Mucopolysaccharidosis type IIIB (MPS IIIB, OMIM #252920), or Sanfilippo syndrome, is caused by a deficiency in the lysosomal enzyme, α-N-acetylglucosaminidase, as a result of mutations in the *NAGLU* gene (O'Brien 1972; Zhao *et al*. 1996). NAGLU deficiency impairs lysosomal degradation of heparan sulphate in the brain, resulting in enlarged lysosomes, secondary storage of gangliosides and cholesterol (Constantopoulos *et al*. 1978; McGlynn *et al*. 2004), severe neuroinflammation (Canal *et al*. 2010; Ohmi *et al*. 2003) and progressive cellular and organ dysfunction (Valstar *et al*. 2008). Patients present with severe behavioural problems including aggression, hyperactivity, a decreased sense of danger and sleep disturbances (Cleary & Wraith 1993; Fraser *et al*. 2005; Moog *et al*. 2007) leading to progressive neurodegeneration and death in the second decade of life, although the phenotype varies considerably (Heron *et al*. 2011; Meyer *et al*. 2007).

There is currently no effective therapy for MPS IIIB, but substrate reduction therapy (Malinowska *et al*. 2009, 2010), enzyme replacement therapy (Yu *et al*. 2000), and gene therapy and stem cell therapy approaches (reviewed in Valstar *et al*. 2008) have been evaluated. Surrogate clinical biomarkers for MPS IIIB have only recently been forthcoming and are still in development (Langford-Smith *et al*. 2010, 2011); therefore, the behavioural phenotype of the mouse model remains an important tool for measurement of treatment effect on the severe neurodegenerative phenotype seen in both mice and patients.

The mouse model of MPS IIIB has previously been reported to show alterations in circadian rhythms as measured in the home cage, including increased activity in the light (Canal *et al*. 2010; Heldermon *et al*. 2007) or dark (Cressant *et al*. 2004). Interestingly, none of these studies, including our own, showed overall increases in activity of MPS IIIB mice. In contrast, an 8-min open field analysis of the MPS IIIB mouse model, half in light, half in dark, showed reductions in activity in both light and dark phases (Li *et al*. 1999), whilst a separate study observed reduced rearing in the second 30 min of a 1-hour open field test (Fu *et al*. 2007). This contrasts with observations of hyperactivity in MPS IIIB mice in a 10-min open field test (Cressant *et al*. 2004).

MPS IIIB mice also show a reduced sense of danger in some situations. Fear conditioning was normal in the contextual fear test but reduced in tone fear test (Li *et al*. 1999) which could be caused by differences in sensory function,

whilst no changes in the acoustic startle response have been observed (Fu *et al*. 2007). A reduced sense of danger (increased entry to open arms) has been observed in the elevated plus maze test when performed in the dark (Cressant *et al*. 2004) but not in the light (Fu *et al*. 2007). Therefore, again it is not clear if the MPS IIIB mice mimic the pathology seen in patients where they have a reduced sense of danger.

These disparities may reflect different testing conditions, and require definitive characterization in both the home cage and open field to determine a reliable phenotype.

Methods

Mouse maintenance

MPS IIIB mice (Li *et al*. 1999) were maintained by heterozygous breeding as described (Canal *et al*. 2010) on a C57BL/6J background with occasional backcrossing to the C57BL/6J line (Harlan, Bicester, UK) to maintain the background. Wild-type (WT) and MPS IIIB knockout (KO) female littermates were used for all behavioural analysis. All procedures were ethically approved and in accordance with the UK Home Office and European Union (2010/63/EU) regulations. Mice had access *ad libitum* to food and water and were housed under a 12-h light–dark cycle.

Behavioural testing

The 1-h open field test was performed as described previously (Malinowska *et al*. 2009, 2010). In brief, 8-month-old female mice ($n = 6$ WT, $n = 6$ MPS IIIB) were dropped into the centre of an arena (260 × 365 mm) containing corn cob flooring and the behaviour was recorded with a digital camera (Sony, Tokyo, Japan) for 60 min. The open field test was performed 1.5 h after the lights were turned on in a randomized blinded fashion. The behaviour was analysed using Top Scan software version 2.0 (Clever Sys. Inc., Reston, VA, USA). The path length, rapid exploratory behaviour (speed >90 mm/second), immobility (speed <0.05 mm/second) and frequency, path length, duration and speed in the central 25% of the cage were analysed.

Rearing was manually counted by an observer blinded to the genotype of the mice. The number of supported rears (using the side of the cage) and unsupported rears were counted for the first 10 min of the 1-h open field.

In the 24-h home cage test, a mouse ($n = 6$ WT, $n = 7$ MPS IIIB) was placed in a cage (260 × 365 mm) with food and a plastic water tower where water is accessible and the mice can nest underneath (Fig. 3a). Behaviour in this area could not be analysed as it is hidden from view. The mice were allowed to acclimatize for 24 h before 24 h of filming. When the lights were turned off, infra red lights automatically turned on to illuminate the mice for the night-vision-enabled camera. Behaviour was analysed as for the 1-h open field using the same measures and Top Scan Suite software (Cleversys Inc., Reston, VA, USA).

The inverted screen test was performed at 8 months ($n = 4$ WT, $n = 5$ MPS IIIB) and 10 months ($n = 3$ WT, $n = 4$ MPS IIIB) of age by placing the mouse on a 470-mm square screen covered with a 13-mm square wire mesh and rotating the screen through 180° over 1–2 seconds. The mouse was suspended upside down above a padded surface and the number of rear leg moves was counted and the amount of time spent suspended up to a maximum of 2 min was recorded.

The horizontal bar test was performed as described previously (Malinowska *et al*. 2010) at 8 months ($n = 6$ WT, $n = 7$ MPS IIIB) and 10 months ($n = 3$ WT, $n = 4$ MPS IIIB) of age. In brief, a 300-mm metal wire, 2 mm in diameter, was secured between two posts 320 mm above a padded surface. The mouse was allowed to grip the centre of the wire and the time to fall or reach the side was recorded, and after 2 min the test was stopped. Crossing the bar in x seconds was scored as $240-x$, remaining on the bar was scored as 120, and falling off the bar after y seconds was recorded as the value of y. The test was repeated three times as a practice run followed by a 10-min rest prior to three tests where the score was recorded.

Statistical analysis

Data were analysed using JMP software version 8 (SAS Institute Inc., Cary, NC, USA) and multivariant analysis of variance (MANOVA) repeated-measure analysis and one-way ANOVAs were used, where appropriate, to determine differences between groups (Genotype) and within groups (Illumination). The F value is given for each comparison, together with degrees of freedom and the probability of error. Significance is assumed at probabilities of less than 0.05.

Results

To test the hypothesis that MPS IIIB mice, like patients, are more hyperactive and have a reduced sense of danger, groups of 6–7, 8-month-old female WT and MPS IIIB mice were monitored for 1 h for habituation behaviour in the open field. To check the reliability of the home cage test, following 24 h of acclimatization, we monitored the same mice in the home cage environment for a further 24 h. We also performed tests of motor function at 8 and 10 months of age to monitor motor decline.

To compare the open field test (Fig. 1a) to previous studies and to determine the minimum time required to achieve consistent significance, we have presented the data as per minute activity graphs and analysed the first 3 and 10 min of the test as well as the total 60 min for significance, using a student's t-test (Cressant *et al*. 2004; Hemsley & Hopwood 2005). MPS IIIB mice travel more than three times as far as WT mice over the duration of the trial (Fig. 1b) with significant differences observed in the first 3 ($F_{1,10} = 13.63$, $P = 0.004$), 10 ($F_{1,10} = 19.69$, $P = 0.001$) and 60 min ($F_{1,10} = 20.70$, $P = 0.001$) of the trial.

Rapid exploratory behaviour was monitored by the frequency (Fig. 1c) and duration (Fig. 1d) of speed over 90 mm/second. Highly significant increases in both of these parameters were observed in MPS IIIB mice at 3, 10 and 60 min after commencement of the test ($F_{1,10} =$ 17.98–33.11, $P = 0.002$–0.0002). Video S1 shows the median mouse from each group over the median 2 min of the open field test (minutes 29–31), running at four times normal speed, in which many of these behaviours can be observed.

We also monitored frequency (Fig. 1e) and duration (Fig. 1f) of immobility over the period of the test. MPS IIIB mice had a consistently reduced frequency and duration of immobility but this was only significant when measured at 10 and 60 min ($F_{1,10} = 16.67$–60.24, $P = 0.008$ to <0.0001) and was not significant at 3 min ($F_{1,10} = 2.99$–1.97, $P = 0.11$–0.19). In these cases, the groups became more significantly different as the analysis period was extended. Interestingly, this appears to be because of an initial similarity in the behaviour of the two groups when the mice were first placed in the cage, which rapidly diverges over time (Fig. 1e,f).

As a prey species, mice tend to display thigmotaxis (Simon *et al*. 1994), and thus show a reduced tendency to enter the central part of an open field arena. In Fig. 2a the frequency of entries into the centre of the open field was recorded every minute for 1 h. Figure 2b shows the path length in the

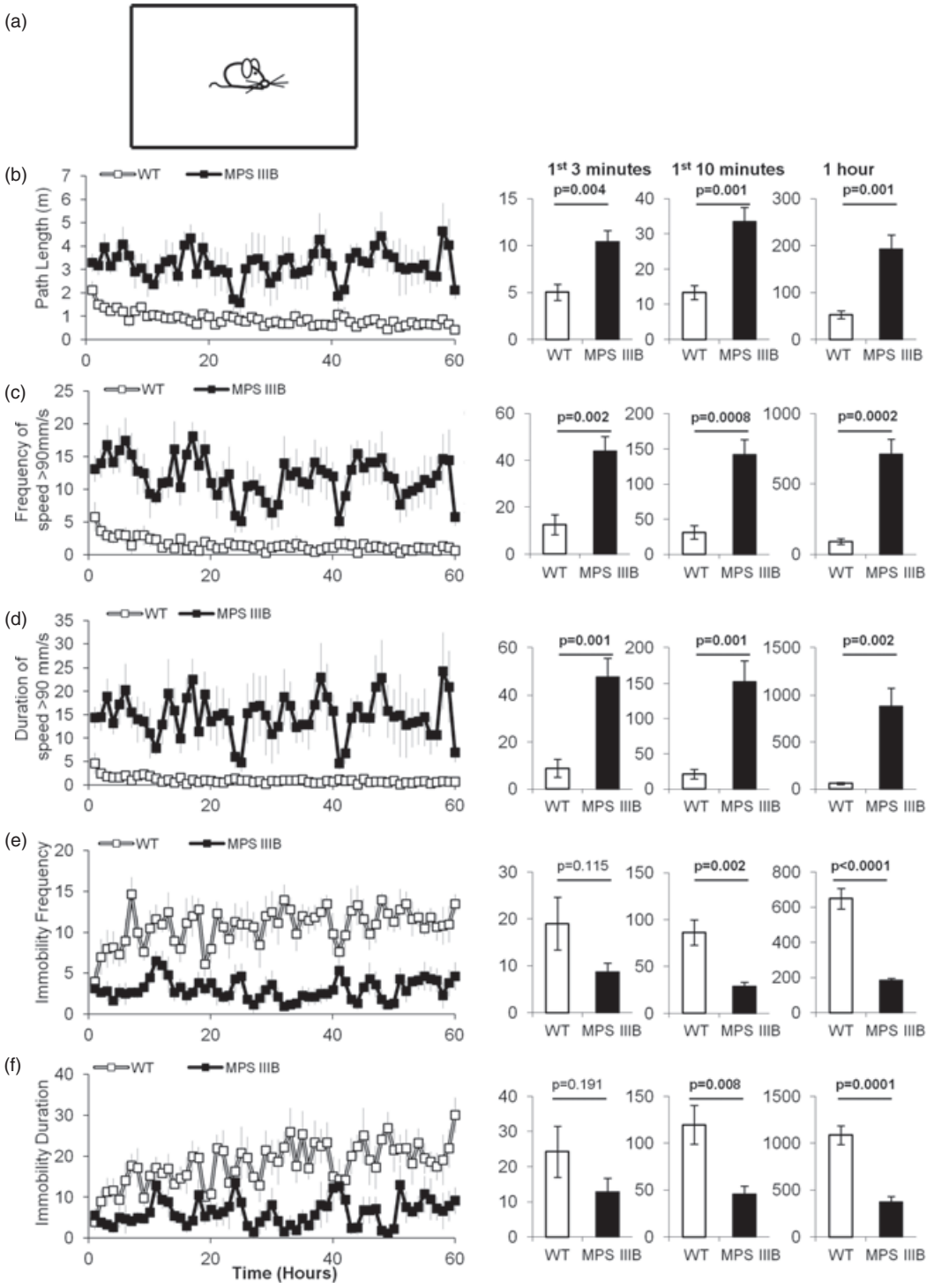

Figure 1: One-hour open field – activity. At 8 months of age six WT (white squares) and six MPS IIIB (black squares) mice were placed in the centre of a cage 260 × 365 mm outlined in (a) and the behaviour was recorded for 1 h. The results of the open field behaviour are presented as a 1-h period with the average of every minute presented and as a bar chart of the first 3 min, first 10 min and whole hour. Error bars represent the standard error of the mean (SEM). *P* values were calculated by *t*-test. The mean average path length in meters (b), frequency of rapid exploratory behaviour (speed >90 mm/second) (c), duration of rapid exploratory behaviour (speed >90 mm/second) (d), frequency of immobility (e) and duration of immobility (f) are presented.

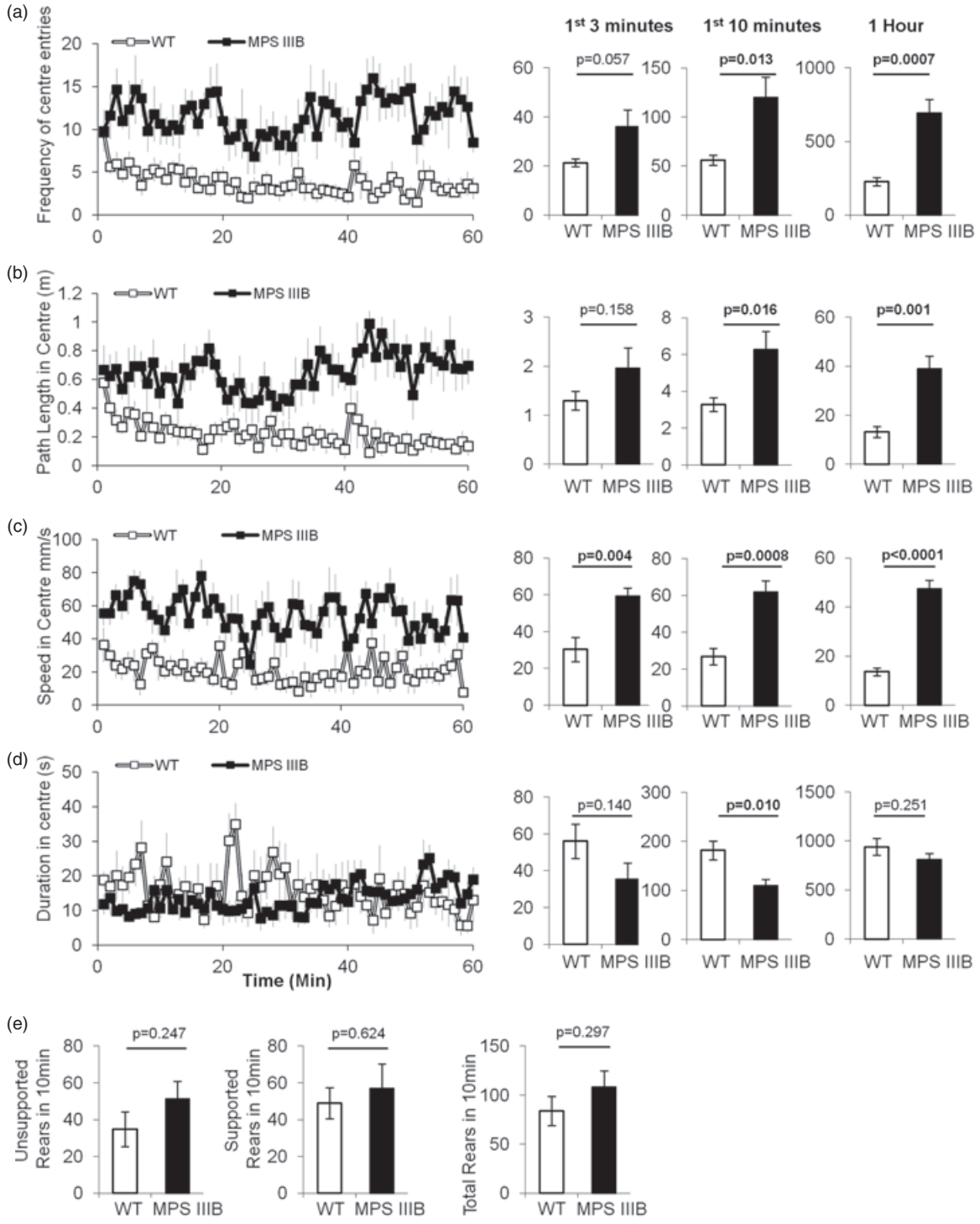

Figure 2: One-hour open field – centre and rearing. At 8 months of age six WT (white squares) and six MPS IIIB (black squares) mice were placed in a cage outlined in Fig. 1a and the behaviour was recorded for 1 h. The results of the open field behaviour are presented as a 1-h period with the average of every minute presented and as a bar chart of the first 3 min, first 10 min and whole hour. Error bars represent the SEM. *P* values were calculated by *t*-test. The frequency of entering the centre (a), path length in the centre of the cage in meters (b), the speed in the centre in millimetre per second (c), time spent in the centre in seconds (d), and the frequency of rearing (e) are presented.

central area and shows a very similar pattern. In the first minute, the WT and MPS IIIB mice are indistinguishable, but diverge in subsequent minutes with the MPS IIIB mice entering the centre more frequently and travelling further. This results in no significant differences between WT and MPS IIIB mice in the first 3 min ($F_{1,10} = 4.63–2.33$, $P = 0.57–0.16$) but a significant increase in MPS IIIB centre entries and path length after 10 min ($F_{1,10} = 8.29–9.04$, $P = 0.02–0.01$) and a greater significant difference after 60 min ($F_{1,10} = 21.19–23.45$, $P = 0.001–0.0007$). MPS IIIB travel at significantly higher speeds in the central area (Fig. 2c) with improving significance from 3 ($F_{1,10} = 13.52$, $P = 0.0043$) to 10 ($F_{1,10} = 22.50$, $P = 0.0008$) to 60 minutes ($F_{1,10} = 78.65$, $P = 0.000005$). This parameter is consistent with the overall rapid exploratory behaviour and may not be related to a reduced sense of danger. Figure 2d shows that there is no clear difference in the duration spent in the centre of the open field, after 3 min there is no significant difference ($F_{1,10} = 2.58$, $P = 0.140$), at 10 minutes the WT mice spent significantly more time in the centre ($F_{1,10} = 10.03$, $P = 0.010$); however, after 60 min there is no significant difference ($F_{1,10} = 1.48$, $P = 0.251$). Both groups of mice spent significantly more time in the sides of the test area than in the centre (not shown) ($F_{1,10} = 296.36$, $P = 9 \times 10^{-9}$); thus, they both display a tendency to thigmotaxis. Overall these data suggest that although MPS IIIB mice enter the central area more frequently and travel further and more rapidly in this area, there is no significant reduction in the sense of danger in MPS IIIB mice in this test in terms of the duration spent within the central area.

Finally, we measured rearing activity of mice over the first 10 min of the open field test (Fig. 2e). This is not measurable using our automated system and was therefore scored by hand. No significant difference was identified in the number of unsupported rears ($F_{1,10} = 1.51$, $P = 0.247$), supported rears using the side of the cage ($F_{1,10} = 0.256$, $P = 0.624$) and total rears ($F_{1,10} = 1.21$, $P = 0.297$) between MPS IIIB or WT mice.

The same groups of female mice were acclimatized in the same test arena as the open field for 24 h by supplying bedding, a house, food and water *ad libitum* (Fig. 3a). Home cage behaviour was then monitored for the subsequent 24 h.

Figure 3b shows the average path length travelled by groups of six to seven WT or MPS IIIB mice in 20-min intervals over 24 h, split into two equal 12-h light or dark periods. All mice have significantly increased path length in darkness than in light (Illumination; $F_{1,11} = 15.46$, $P = 0.002$) and over 24 h, MPS IIIB mice have a significant increase in path length over their WT counterparts, travelling on average 656 m compared to 298 m for WT mice (Genotype; $F_{1,11} = 5.29$, $P = 0.03$). However, there are no significant differences between MPS IIIB and WT when measured only in darkness (Genotype; $F_{1,11} = 2.56$, $P = 0.138$) or light (Genotype; $F_{1,11} = 3.22$, $P = 0.100$). This suggests that a minimum of 24 h observation in the home cage is required to show significant differences in path length.

Rapid exploratory behaviour is measured as frequency (Fig. 3c) and duration (Fig. 3d) of speed over 90 mm/second as before. All mice have an increased frequency of speed over 90 mm/second in darkness compared to light (Illumination; $F_{1,11} = 9.23$, $P = 0.013$). MPS IIIB mice have a borderline significantly increased frequency of speed over 90 mm/second over the entire 24-h period (Genotype; $F_{1,11} = 4.05$, $P = 0.057$) and an almost significant difference between genotypes when this is measured in light (Genotype; $F_{1,11} = 4.27$, $P = 0.063$) but not in darkness (Genotype; $F_{1,11} = 2.48$, $P = 0.144$) alone, because of the increased variability in the dark. Interestingly the duration of speed over 90 mm/second showed that MPS IIIB mice spent significantly less time travelling faster than 90 mm/second over 24 h (Genotype; $F_{1,11} = 7.91$, $P = 0.01$) and in the dark (Genotype; $F_{1,11} = 8.80$, $P = 0.01$), which is neither consistent with the frequency and the path length nor the results of the 1-h open field test.

The frequency (Fig. 3e) and duration (Fig. 3f) of immobility were also recorded over 24 h. Both the frequency (Illumination; $F_{1,11} = 54.24$, $P = 0.00001$) and duration (Illumination; $F_{1,11} = 9.32$, $P = 0.004$) of immobility were significantly increased in darkness for all mice and overall MPS IIIB mice had increased frequency (Genotype; $F_{1,11} = 4.42$, $P = 0.047$) and duration (Genotype; $F_{1,11} = 9.69$, $P = 0.005$) of immobility. For duration of immobility we observed a significant genotype effect in light (Genotype; $F_{1,11} = 9.32$, $P = 0.011$) and dark (Genotype; $F_{1,11} = 4.67$, $P = 0.054$).

In the 24-h home cage test the overall frequency of entering the centre (Fig. 4a) was significantly higher in the dark (Illumination; $F_{1,11} = 17.46$, $P = 0.0015$) and there was an overall increase in centre entries by MPS IIIB mice (Genotype; $F_{1,11} = 6.96$, $P = 0.015$). In light there is also a significant effect (Genotype; $F_{1,11} = 6.52$, $P = 0.027$). This parameter is consistent with the hyperactivity observed in the 24-h home cage test and 1-h test. The distance travelled in the centre (Fig. 4b) is also consistent with hyperactivity as there is a significant overall increase in MPS IIIB path length in the centre (Genotype; $F_{1,11} = 6.14$, $P = 0.021$) and in the light (Genotype; $F_{1,11} = 5.21$, $P = 0.043$) but not in the dark (Genotype; $F_{1,11} = 2.98$, $P = 0.112$). There is also increased activity for all mice in the dark compared to light (Illumination; $F_{1,11} = 7.10$, $P = 0.017$). The speed in the centre is likewise consistent with hyperactivity with a significant overall genotype (Genotype; $F_{1,11} = 5.71$, $P = 0.026$) and illumination effect (Illumination; $F_{1,11} = 8.31$, $P = 0.015$). However, the increased speed in the centre observed in MPS IIIB mice only occurs in the light (Genotype; $F_{1,11} = 40.99$, $P = 0.00005$) and not in the dark (Genotype; $F_{1,11} = 0.16$, $P = 0.69$). There is a significant illumination difference in the duration of time spent in the centre (Fig. 4d) (Illumination; $F_{1,11} = 12.69$, $P = 0.0045$) but no genotype difference. This suggests that there is no detectable difference in sense of danger between MPS IIIB and WT mice.

To assess loss of motor skills and muscle strength with time, we firstly used the inverted screen test over 120 seconds (Fig. 5a) to assess the number of rear leg moves performed (Fig. 5b) and the duration to fall over this period (Fig. 5c). At 8 months of age, WT mice perform on average almost twice as many rear leg movements as MPS IIIB and also fall later than MPS IIIB mice, but neither effect is significant because of a large variability (Moves: $F_{1,11} = 1.53$, $P = 0.241$; Time: $F_{1,11} = 1.05$, $P = 0.327$). At 10 months of age

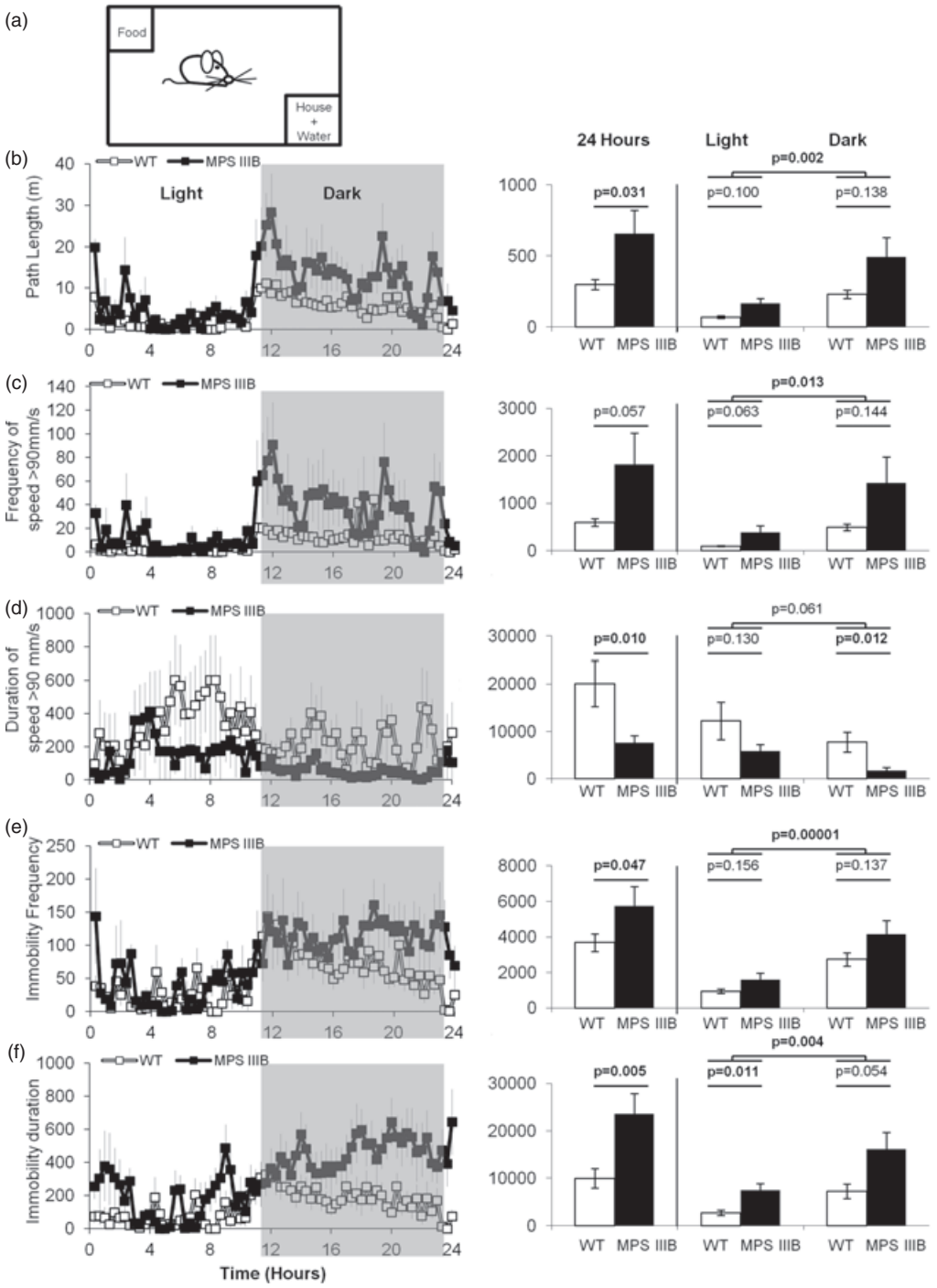

Figure 3: Home cage behaviour – activity. At 8 months of age six WT (white squares) and seven MPS IIIB (black squares) mice were placed in a cage 260 × 365 mm outlined in (a) that included a food bowl and nesting house containing water within it. After 24 h of acclimatization the home cage behaviour was recorded for 24 h using infrared lights for night-time monitoring. The results of the home cage behaviour are presented as a 24-h period with the average of every 20 min presented and as a bar chart of firstly the whole 24 h and secondly the light and dark periods separately. Error bars represent the SEM. *P* values were calculated by MANOVA repeated-measure analysis and one-way ANOVA. The mean average path length in meters (b), frequency of rapid exploratory behaviour (speed >90 mm/second) (c), duration of rapid exploratory behaviour in seconds (d), frequency of immobility (e) and duration of immobility (f) are presented.

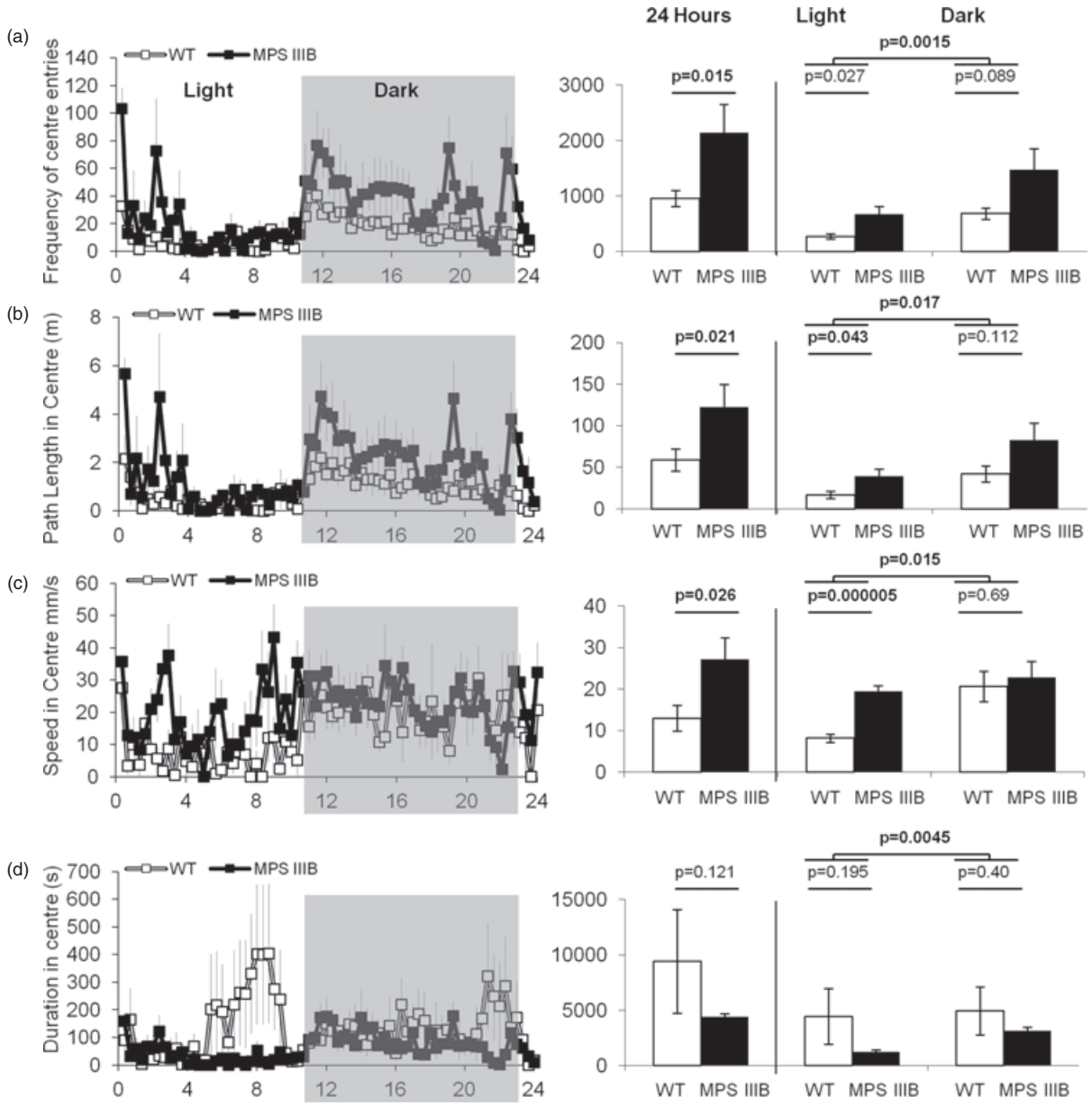

Figure 4: Home cage behaviour – centre. At 8 months of age six WT (white squares) and seven MPS IIIB (black squares) mice were placed in a cage outlined in Fig. 3a, after 24 h of acclimatization the home cage behaviour was recorded for 24 h. The results of the home cage behaviour are presented as a 24-h period with the average of every 20 min presented and as a bar chart of firstly the whole 24 h and secondly the light and dark periods separately. Error bars represent the SEM. *P* values were calculated by MANOVA repeated-measure analysis and one-way ANOVA. The frequency of entering the centre (a), path length in the centre of the cage in meters (b), the speed in the centre in millimetre per second (c) and time spent in the centre in seconds (d) are presented.

the number of rear leg movements ($F_{1,5} = 5.80$, $P = 0.06$) and the time to fall from the screen ($F_{1,5} = 5.86$, $P = 0.06$) were on the borderline of a significant decrease in MPS IIIB mice. Increased group size would therefore probably have shown a significant effect in both parameters at 10 months.

The horizontal bar test measures motor co-ordination and grip strength (Fig. 5d). At 8 months of age there is no significant difference between MPS IIIB and WT mice ($F_{1,11} = 2.34$, $P = 0.15$) (Fig. 5e), but by 10 months of age the MPS IIIB mice are significantly worse at crossing the bar than WT mice ($F_{1,5} = 25.13$, $P = 0.004$). This is in agreement with the inverted screen test and suggests that the mice continue to display normal motor skills at 8 months, which are beginning to decline by 10 months of age.

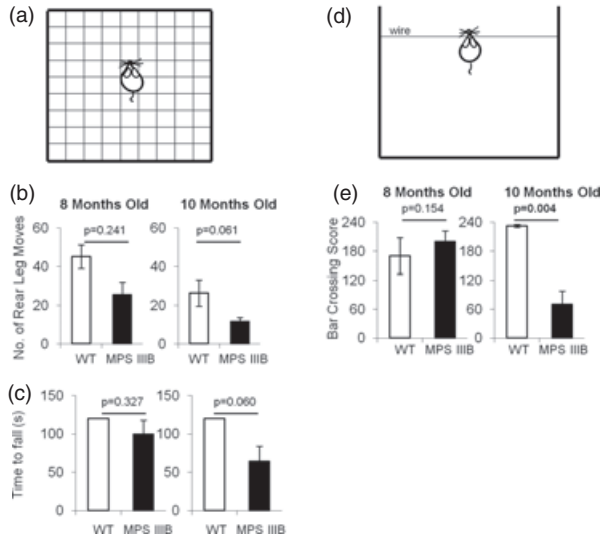

Figure 5: Inverted screen and horizontal bar crossing. At 8 months of age four WT and five MPS IIIB mice and at 10 months of age three WT and four MPS IIIB mice performed the inverted screed test (a). The number of rear leg moves on the inverted screen (b) and the time spent on the inverted screen (c) were recorded. At 8 months of age six WT and seven MPS IIIB mice and at 10 months of age three WT and four MPS IIIB mice performed the horizontal bar test (d). The horizontal bar score (e) is a measure of the time of the time taken to cross or fall from the bar. Error bars represent the SEM and *P* values were calculated by *t*-test.

Discussion

In this work we have been able to determine several behavioural parameters that allow analysis of treatment effects on the neurological deterioration seen in MPS IIIB mice.

The 1-h open field habituation test performed at the same time of day gives clear genotype separation at 10 min and to a greater extent at 1 h. Eight-month-old MPS IIIB mice show a significantly increased path length, frequency and duration of rapid exploratory behaviour and reduced frequency and duration of immobility, all of which are consistent with a hyperactive phenotype. They also show significantly increased frequency of entry, path length and speed in the central area whilst duration in the centre was unchanged. This suggests that these mice do not have a reduced sense of danger when measured in this test, although the relatively small size of the arena used in this study could have reduced this effect (Crawley 2007). The difference between WT and MPS IIIB mouse behaviour often appeared to diverge from a similar starting point in the first minute or two of the tests which led to non-significant differences when observations were measured in the first 3 min of the test, as is used by some researchers in the field (Hemsley & Hopwood 2005). Although all parameters were significant when measured over the first 10 min of the test (with the exception of duration in centre) as is used by many researchers (Cressant *et al*. 2004), seven of eight parameters were more consistently significantly different when measured over the entire 1-h test period.

Fu *et al*. performed a 1-h open field test on 4.5–5-month-old male and female mice, but did not report activity or anxiety differences except for a decrease in rearing in the second 30 min by male mice (Fu *et al*. 2007). It is possible that 4.5–5 months of age is too early to detect differences in genotypes but it is also critical that the test is performed at the same time of day and this is not noted in the study. We did not observe differences in any type of rearing activity between 8-month-old MPS IIIB and WT mice; however, this was only measured over the first 10 min. In a 10-min open field test (Cressant *et al*. 2004), performed at 5 weeks and 7 months of age, no significant genotype differences were observed at 5 weeks of age but significant hyperactivity was seen in MPS IIIB mice at 7 months, in line with the results presented here, which is also consistent with the patient phenotype (Cleary & Wraith 1993).

MPS IIIB mice showed increased frequency of entry, path length and speed in the central area whilst duration in the centre was unchanged. These increases can all be explained by increased hyperactivity causing the mice to move faster and so entering the centre more frequently and travelling further in the same amount of time. MPS IIIB mice have been showed to be less anxious when using the elevated plus maze (Cressant *et al*. 2004); however, significant changes were only seen in darkness, and no anxiety differences were seen in a 1-h open field test in daylight (Fu *et al*. 2007). These discrepancies could be because of the relatively small size of the open field used in our studies (Crawley 2007), or the effect seen in darkness could be an artefact (Cressant *et al*. 2004). To test this hypothesis a larger open field test arena would be required.

In the 24-h test we observed significant genotype effects in all parameters except duration in the centre; however, significance was much less evident compared to the 1-h test, and increases in immobility frequency and duration in MPS IIIB mice in the 24-h test are not consistent with the hyperactive behaviour seen in the 1-h open field test. Differences between MPS IIIB and WT mice appeared to be more evident in daylight than in darkness where few changes were seen. This could be because mice are usually less active in daylight; thus, hyperactive behaviour is more easily distinguished during a relatively inactive phase. Interestingly, children with Sanfilippo are reported to have circadian rhythm disturbances, with difficulty in sleeping and frequent night waking and night wandering (Fraser *et al*. 2002, 2005), which would be consistent with the phenotype observed here. In addition, significant periodicity was noted in MPS IIIB mice during the dark phase of the 24-h test, which may have confounded the ability to distinguish genotypes in darkness. Similar MPS IIIB periodicity, and, in particular, a late bout of activity at the transition between light and dark were previously observed by us in a circadian analysis of MPS IIIB mice (Canal *et al*. 2010). MPS IIIB mice show increased frequency and duration of all activity measures with commensurate reductions in frequency and duration of immobility in the 1-h open field, which is consistent

with sustained hyperactive behaviour. However, in the home cage, although path length and frequency of rapid exploratory behaviour are increased, other measures such as duration of rapid activity are not. MPS IIIB mice also have increased frequency and duration of immobility in the home cage. This discrepancy could reflect the fact that there is a nesting box in the home cage where behaviour could not be measured, but this is unlikely as there is no change in the duration of time spent in this area between genotypes. It could instead reflect different types of hyperactive behaviour during exploratory behaviour while the mouse habituates to a novel environment in the 1-h test compared to normal behaviour in the home cage. The 24-h home cage behaviour suggests that the mouse is not able to sustain rapid activity over longer periods, and is compensating by spending more time immobile.

The 24-h data broadly support the previous circadian analysis by Heldermon *et al*. and ourselves (Canal *et al*. 2010; Heldermon *et al*. 2007, 2010), but is less effective at separating the phenotypes and less convincingly shows a hyperactive phenotype. Cressant *et al*. also performed a home cage test over a 24-hour period; however, that study lacked a 24-hour acclimatization period, so has an element of habituation (Cressant *et al*. 2004). They observed no difference between MPS IIIB and WT mice in the light but a significant increase in the time the MPS IIIB mice spent in motion in the dark at 4.5 months of age. The open field test could be more consistent than the 24-h home cage test because the circadian time that testing was performed at (0830 h) is close to a peak of activity that we (Canal *et al*. 2010) have previously observed in MPS IIIB mice just before the lights were switched on in the early morning (0700 h). This peak can be seen in many of the 24-h measures in these data as well. In contrast, WT mice are becoming less active as the lights come on. The inverted screen test did not show a significant effect at 8 or 10 months of age but there was a clear trend towards the MPS IIIB mice moving less and being less able to grip the inverted screen. A larger *n* number for this experiment may make this effect significant. There is a clear significant failure of MPS IIIB mice to complete the horizontal bar crossing test at 10 months of age but no changes at 8 months of age. Taken together these changes suggest that motor skills are lost late by MPS IIIB mice at 10 months of age, and that it can be variable between mice. A rocking rotarod test was performed by Heldermon *et al*. which also measures motor skills, and a significant defect in MPS IIIB mice was seen from 9 months onwards which supports the inverted screen and horizontal bar test results (Heldermon *et al*. 2007, 2010). An accelerating rotarod test was not reported to show a significant difference between WT and MPS IIIB mice at 5 months of age (Fu *et al*. 2007, 2010) or from 1.5 to 13 months of age (Heldermon *et al*. 2007). In our hands motor skills do not deteriorate significantly until 9–10 months of age; thus, the horizontal bar test and the inverted screen test only have utility for the long-term evaluation of therapies when the mice are at least 9–10 months of age and group sizes are more than 6–7. In addition, the observation of urinary retention in these mice from 8 to 10 months of age onwards (Malinowska *et al*. 2010) is clearly a limiting factor in their ability to perform motor tasks and as such the use of motor tests after 8–9 months of age is more likely to measure this effect than a central dysfunction in motor abilities.

In our previous work on treating MPS IIIB mice with genistein we found similar hyperactivity increases in male MPS IIIB mice, although differences were less marked than females (Malinowska *et al*. 2010). Although this would suggest that female mice may be more consistent for behavioural testing, Fu *et al*. (2007) reported vertical activity changes only observed in males (Fu *et al*. 2007). The ability to readily group house females makes them a more economic proposition than males.

Conclusion

We have showed that it is possible to detect consistently increased hyperactivity and rapid exploratory behaviour in the mouse model of MPS IIIB at 8 months of age using a 1-h open field test and modest group sizes of 6–7, which is consistent with MPS IIIB patient behaviour. The minimum test time that we would recommend for open field behaviour is 10 min, with more consistent data obtained at 60 min, and it may be important that tests are carried out close to the time at which lights are switched on in the morning. Home cage behaviour by contrast is a much less reliable distinguisher of phenotype in MPS IIIB mice and may reflect less sustained hyperactive behaviour. Finally, motor skill evaluations are not likely to yield useful data before 9–10 months of age and can be significantly influenced by gait impairment as a result of urinary retention. This simple protocol will allow the evaluation of potential therapies for MPS IIIB and could prove useful in the development of assessment protocols for other mouse models of neurodegenerative diseases.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Video S1: One-hour open field. A segment of video recorded during the 1-h open field test, which shows the median mouse from each group (MPS IIIB on the left, WT on the right) over the median 2 min of the test (minutes 29–31). The clip is shown at four times normal speed.

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Female Mucopolysaccharidosis IIIA Mice Exhibit Hyperactivity and a Reduced Sense of Danger in the Open Field Test

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Abstract

Reliable behavioural tests in animal models of neurodegenerative diseases allow us to study the natural history of disease and evaluate the efficacy of novel therapies. Mucopolysaccharidosis IIIA (MPS IIIA or Sanfilippo A), is a severe, neurodegenerative lysosomal storage disorder caused by a deficiency in the heparan sulphate catabolising enzyme, sulfamidase. Undegraded heparan sulphate accumulates, resulting in lysosomal enlargement and cellular dysfunction. Patients suffer a progressive loss of motor and cognitive function with severe behavioural manifestations and premature death. There is currently no treatment. A spontaneously occurring mouse model of the disease has been described, that has approximately 3% of normal enzyme activity levels. Behavioural phenotyping of the MPS IIIA mouse has been previously reported, but the results are conflicting and variable, even after full backcrossing to the C57BL/6 background. Therefore we have independently backcrossed the MPS IIIA model onto the C57BL/6J background and evaluated the behaviour of male and female MPS IIIA mice at 4, 6 and 8 months of age using the open field test, elevated plus maze, inverted screen and horizontal bar crossing at the same circadian time point. Using a 60 minute open field, we have demonstrated that female MPS IIIA mice are hyperactive, have a longer path length, display rapid exploratory behaviour and spend less time immobile than WT mice. Female MPS IIIA mice also display a reduced sense of danger and spend more time in the centre of the open field. There were no significant differences found between male WT and MPS IIIA mice and no differences in neuromuscular strength were seen with either sex. The altered natural history of behaviour that we observe in the MPS IIIA mouse will allow more accurate evaluation of novel therapeutics for MPS IIIA and potentially other neurodegenerative disorders.

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Introduction

Mucopolysaccharidosis IIIA (MPS IIIA, OMIM #252900), or Sanfilippo Type A, is an autosomal recessive lysosomal storage disorder that affects 0.82 in 100,000 live births in the United Kingdom [1]. The disease is characterised by severe and progressive loss of cognitive and motor functions, behavioural difficulties and eventually death in the second decade of life, although the severity and progression of the disease varies widely [1,2,3]. MPS IIIA is caused by mutations in the *SGSH* gene that result in deficiency of the N-sulfoglucosamine sulfohydrolase enzyme (sulfamidase, EC 3.10.1.1) and subsequent accumulation of undegraded heparan sulphate, lysosomal enlargement and cellular and organ dysfunction [4,5,6,7]. Patients exhibit progressive neurodegeneration and behavioural problems including hyperactivity, a reduced sense of danger, aggression and sleep disturbances [7,8,9,10,11,12].

Although there are no current therapies, several strategies are in development for MPS IIIA or the phenotypically indistinguishable MPS IIIB disease, including substrate reduction therapy [13,14,15], intrathecal enzyme replacement therapy [16,17,18,19,20] and gene therapy with lentiviral [21], adenoviral [22] or adeno-associated-viral [23] vectors. Most strategies make use of the ability of exogenous enzyme to complement affected cells, however the presence of the blood brain barrier limits efficient enzyme distribution. Biomarkers for MPS IIIA or related diseases are still in development [24,25], thus the accurate evaluation of neurodegeneration using behavioural phenotyping in the mouse model of disease is paramount. A spontaneously occurring mouse model of MPS IIIA on a mixed 129SvJ, C57BL/6, SJL, and CD1 background has been previously described with around 3% of normal enzyme activity and exhibiting many of the features observed in patients [26]. The MPS IIIA mice exhibit severe neuropathology characterised by enlarged lysosomes, primary storage of HS, secondary storage of GM2 and GM3 gangliosides and cholesterol and chronic neuroinflammation [19,23,26,27,28]. The MPS IIIA mice are euthanized between 9 and 12 months of age due to urine

retention, a phenotype not seen in the patients but also seen in MPS IIIB mice [29]. The MPS IIIA mouse model has been backcrossed to the C57BL/6 background by Professor John Hopwood's Lysosomal Diseases Research Unit [30] and to the C57BL/6J background by Jackson laboratories [31].

Although the behaviour of the MPS IIIA mouse model has been evaluated in the open field test at several ages, using different sexes, by several groups and on different strain backgrounds, there is a remarkable amount of variability in the outcome of the test as outlined in Table 1.

Using the mixed background mouse, at many of the time points measured, no differences are observed between either male or female WT and MPS IIIA mice, with initial findings of hyperactivity and later hypoactivity in males [18,32]. After back crossing the MPS IIIA mice on to a C57BL/6 background, the behaviour was extensively studied by Crawley *et al.* at different time points. Hyperactivity in males was observed at 12 weeks of age, and females at 22 and 32 weeks of age, although these mice were not naively tested [30]. At most time points, no differences were seen. However male, but not female, MPS IIIA mice were shown to be hypoactive at some time points in the work of Lau *et al* [33] and male MPS IIIA mice were hypoactive at 15 weeks of age in a more recent paper by Lau *et al.* [34]. Hypoactivity in male mice was also observed at some time points in McIntyre *et al.* [21]. In columns 7, 8, 9 and 10 of Table 1 (marked as bold) we have also presented markedly variable outcomes of observations of locomotor behaviour of MPS IIIA and WT mice that received control intracranial injections [18,21,23,35].

Patients with MPS IIIA are believed to have a reduced sense of danger, which can be inferred in mice by the amount of time spent in the centre of the open field test or by use of the elevated plus maze test, both of which can be used to measure their tendency to avoid open spaces and remain close to cover (thigmotaxis). No difference in the time in centre of the open field was detected by Lau *et al.* [33], however this could be due to the small size of the open field used. In the elevated plus maze, male MPS IIIA mice were reported to display reduced anxiety with a greater proportion of their path length (distance travelled) spent in the open arms at some time points but not others [33,34].

Overall, there are discrepancies in behaviour of this mouse model that could be due to gender differences, the age of testing and the methodology used to perform the tests. Therefore we have attempted to perform a standardised analysis of MPS IIIA mice by first backcrossing them to the C57BL/6J background for over 10 generations and subsequently testing a cohort of MPS IIIA and WT mice at the same circadian time point at 4, 6 and 8 months (16, 24 and 32 weeks) of age in the 60 minute open field test, the elevated plus maze, as well as several other neuromuscular evaluations that we have previously shown to be effective for phenotyping MPS IIIB mice [36]. The female MPS IIIA mice were hyperactivity, had a recued sense of danger and no neuromuscular differences. The open field test performed at the same point in the circadian rhythm was a consistent, sensitive and reliable behavioural test for the evaluation of novel therapeutic strategies in MPS IIIA mice.

Methods

Mouse maintenance

The MPS IIIA mouse colony was maintained through heterozygous breeding at the University of Manchester, all procedures were ethically approved by the University of Manchester Ethical Review Process Committee and in accordance with the UK Home Office regulations under project licence PPL 40/3117. Mice were housed in individually ventilated cages, had access *ad libitum* to food and water and were in a 12 hour light and dark cycle. Male mice were singly housed at 14 weeks of age due to aggressive tendencies but female mice remained housed in groups of 4–6. The MPS IIIA mice (B6.Cg-Sgsh^{mps3a}) have been backcrossed from the original mixed 129SvJ, C57BL/6, SJL, and CD1 background [26] onto the C57BL/6J background by more than 10 generations of backcrossing with C57BL/6J mice (Harlan, UK) and were maintained by heterozygous breeding. WT and MPS IIIA littermates have been used in all behavioural experiments.

Genotyping MPS IIIA mice

MPS IIIA mice have a G to A mutation in the *SGSH* gene which removes a *Msp*1I restriction enzyme digestion site. Genotyping is performed by PCR amplification of DNA followed by *Msp*1I digestion and observation of the size of DNA fragments produced. Genomic DNA was extracted from ear punches using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma Aldrich) following the manufacturer's instructions and *SGSH* was amplified by PCR using the forward primer 5′ GTGTTCCCTGCCTGCTCAC 3′ and reverse primer 5′ CCAGTCCCCTCATCCCACTA 3′. The DNA was digested with *Msp*1I (New England Biolabs, UK) and the DNA fragments were separated by 2% agarose gel electrophoresis. The genotype was determined from the pattern of DNA fragments; wild type (WT) reveals 199 bp, 118 bp and 78 bp fragments, mutant (MUT) 317 bp and 78 bp fragments and heterozygous (HET) 317 bp, 199 bp, 118 bp and 78 bp.

Behavioural testing

At 4, 6 and 8 months (16, 24 and 32 weeks) of age, the same cohorts of 10 male WT and 10 male MPS IIIA mice and 10 female WT and 11 MPS IIIA mice were analysed with the following behavioural tests. 1.5 hours into the 12 hour light phase the mice were dropped into the centre of an open field arena (width 450 mm, depth 450 mm, height 500 mm) made of matt white acrylic. The behaviour was recorded for 60 minutes using a digital camcorder (Sony) and analysed using Top Scan software (Clever Sys. Inc., USA). The path length, frequency (number of times) and duration of rapid exploratory behaviour (speed $>$100 mm/s), frequency and duration of immobility (speed $<$5 mm/s) and frequency of entering the centre and duration in the centre (75 mm from each edge) was analysed. Rearing in the open field was analysed by counting the number of unsupported rears (front paws off the floor) and supported rears (front paws on the wall) in the first 10 minutes. The same researcher performed all experiments and was blinded to genotype.

After a 30 minute rest, the mice were placed onto the end of an open arm of an elevated plus maze. The maze was constructed of matt white acrylic, comprised of four 500 mm long by 100 mm wide arms, two of which were enclosed by 500 mm high walls, and was raised on a stand 500 mm off the floor. Mice were placed so they faced towards the centre of the maze and were given 10 minutes to explore the maze before returning to a cage. The maze was cleaned using 70% ethanol. 30 minutes later the mice were tested for a second time, in the same manner. The results were analysed using Top Scan software (Clever Sys) to examine the amount of time spent on the open arm, the percentage of path length in the open arm, and the percentage of open arm entries.

30 minutes later the inverted screen test was performed, as described previously [36]. In brief, at 4, 6 and 8 months of age the mouse was placed on a 470 mm square with a 13 mm square wire mesh. The screen was then rotated through 180° over 1–2 seconds. The mouse was then suspended upside down over a

Table 1. Summary of the significant differences in the open field activity of MPS IIIA mice in the literature.

Paper		Hemsley *et al.*		Hemsley *et al.*	Lau *et al.*		Lau *et al.*	McIntyre *et al.*	Crawley *et al.*		* *Hemsley et al.*	* *Hemsley et al.*	* *McIntyre et al.*	* *Fraldi et al.*	This Study	
Year		2005		2007	2008		2010	2010	2006		*2007*	*2009*	*2010*	*2007*		
Background		Mixed		Mixed	C57BL/6		C57BL/6	C57BL/6	C57BL/6		*Mixed*	*C57BL/6*	*C57BL/6*	*C57BL/6*	C57BL/6J	
Measure		Line Crossing		Line Crossing	Zone Entries		Path Length	Line crossing	Line Crossing		*Line Crossing*	*Path Length*	*Line crossing*	*Path Length*	Path Length	
Sex		Male	Female	Male	Male	Female	Male	Male	Male	Female	*Male*	*Male*	*Male*	*Male*	Male	Female
Age (Weeks)	**3**	Hyper	Hyper		NS	NS										
	4								NS	Hypo						
	5				NS	NS										
	6	Hypo	NS	Hypo					NS	NS						
	8								NS	NS	*NS* +*Hypo**					
	10	NS	NS		Hypo	NS			NS	NS				*NS**		
	12								Hyper	NS	*NS* +*Hypo**	*Hypo**				
	15	Hypo	NS		NS	NS	Hypo		NS	NS				*NS**		
	16														NS	Hyper
	18				Hypo	NS			NS	NS	*Hypo**			*NS**		
	20	NS	NS					Hypo					*NS**			
	21													*Hyper**		
	22				Hypo	NS			NS	Hyper						
	24							NS					*NS**		NS	Hyper
	25								NS	NS						
	28							NS					*NS**			
	32								NS	Hyper					NS	Some Hyper
	40	NS	NS						NS	NS						

Hyper indicates significant hyperactive behaviour and hypo indicates significant hypoactive behaviour in the open field test. NS indicates no significant difference and * indicates mice that have recieved intracranial injections. Control injections contained: 2.5 μl of 50 mM sodium acetate, 100 mM sodium chloride at pH 5.0 in Hemsley *et al* 2007, 4 μl 10 mM sodium phosphate, 140 mM sodium chloride pH 7.0 in Hemsley *et al*. 2009, 5 μl 0.9% (w/v) sodium chloride in McIntyre *et al*. 2010 and 1 μl GFP adeno-associated viral vector in Fraldi *et al*. 2007. The work of Hemsley et *al*. 2007 contained multiple control treated groups and where there were differences between groups, both have been shown. Where female mice are not mentioned or cells are blank no testing was performed.

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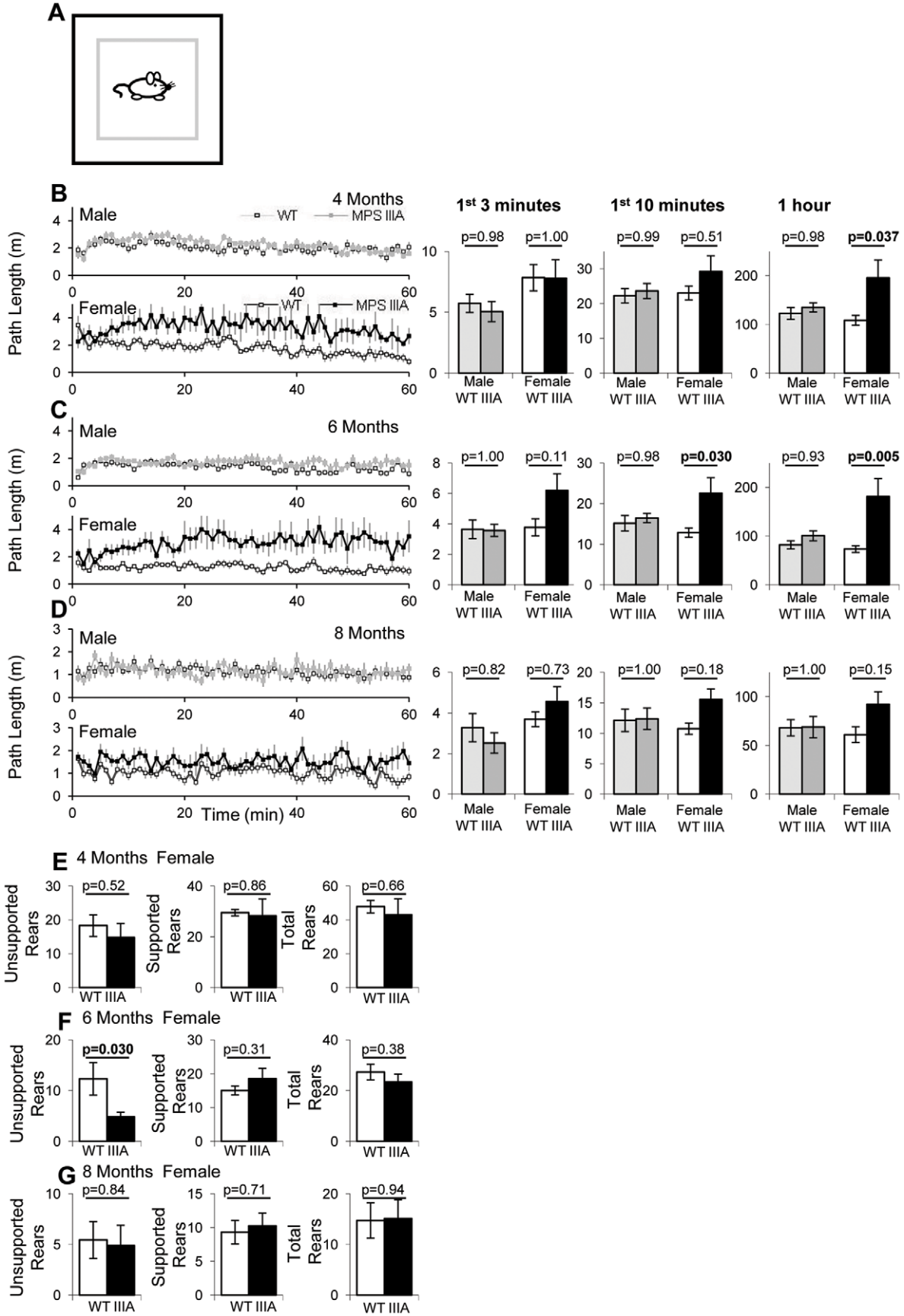
A
B
4 Months
Male
WT
MPS IIIA
Female
Path Length (m)
1st 3 minutes
1st 10 minutes
1 hour
p=0.98
p=1.00
p=0.99
p=0.51
p=0.98
p=0.037
Male WT IIIA
Female WT IIIA
C
6 Months
p=1.00
p=0.11
p=0.98
p=0.030
p=0.93
p=0.005
D
8 Months
p=0.82
p=0.73
p=1.00
p=0.18
p=1.00
p=0.15
Time (min)
E
4 Months Female
Unsupported Rears
Supported Rears
Total Rears
p=0.52
p=0.86
p=0.66
WT IIIA
F
6 Months Female
p=0.030
p=0.31
p=0.38
G
8 Months Female
p=0.84
p=0.71
p=0.94

Figure 1. Open field path length and rearing. At 4, 6 and 8 months (16, 24 and 32 weeks) of age, 10 WT male (light grey squares), 10 MPS IIIA male (dark grey squares), 10 WT female (white squares) and 11 MPS IIIA female (black squares) were placed in the open field and the behaviour was recorded for 60 minutes (A). The results of the open field behaviour are presented as a 60 minute period with the average of every minute presented and as a bar chart of the first 3 minutes, first 10 minutes and the whole hour. Error bars represent the standard error of the mean (SEM). p values were calculated by 2 Way ANOVA. The mean average path length in Metres at 4 (B), 6 (C) and 8 (D) months have been presented. The number of unsupported, supported and total rears in the first 10 minutes at 4 (E), 6 (F) and 8 (G) months of age have also been presented.
doi:10.1371/journal.pone.0025717.g001

padded surface; the rear leg moves were counted and the duration suspended was recorded up to 2 minutes.

One hour later the horizontal bar test was performed as described previously [14,36]. In brief, a 2 mm diameter, 300 mm long metal wire was secured between 2 posts, 320 mm above a padded surface. The mouse was allowed to grip the centre of the wire and the time taken to fall or to reach the side was recorded up to 2 minutes. The test was repeated three times as a training run followed by a 10 minute rest before three test runs. The results were scored as follows: crossing the bar in x seconds was scored as 240-x, remaining on the bar was scored as 120 and falling off the bar after y seconds was recorded as y.

Urine retention

Upon sacrifice 9 male WT, 8 male MPS IIIA, 7 female WT and 8 female MPS IIIA mice at 8 months (32 weeks) of age were dissected and the urine was removed from the bladder and volume measured using an insulin syringe (BD).

Statistical analysis

Statistical analysis was performed using JMP software (SAS Institute Inc, Cary, NC, USA) and analysed by MANOVA with repeated measures for overall significances and by one or two way ANOVA, as appropriate, with Tukey post hoc tests at individual time points. Significance was set at $p \leq 0.05$. For the MANOVA analysis the Genotype significance determines if there is a difference between WT and MPS IIIA mice irrespective of the age of the mice, the Time significance determines if there is a change in the behaviour of the mice at different ages irrespective of the genotype and the Genotype*Time significance determines if MPS IIIA mouse behaviour changes over time in a different manner to WT mice.

Results

Cohorts of male and female, WT and MPS IIIA mice (n = 10–11 per group) were monitored in a 60 minute open field test as outlined in Figure 1A. The path length minute by minute over 60 minutes is shown at 4 months (16 weeks) in Figure 1B, 6 months (24 weeks) in Figure 1C and at 8 months (32 weeks) in Figure 1D. The behaviour in the first 3 minutes, 10 minutes and 60 minutes is presented to allow comparison against published data and to identify the minimum amount of time required for statistically significant analyses. At 4 months of age, female MPS IIIA mice were significantly hyperactive and travel almost twice as far as the WT mice over 60 minutes ($p = 0.037$; Figure 1B). Examining the behaviour over the first 3 minutes showed no difference and no trend in female behaviour, but over 10 minutes there was a non-significant trend towards hyperactivity. No difference was found in the path length of male WT or MPS IIIA mice at 4 months.

At 6 months of age the same female MPS IIIA mice demonstrated increased path length with significant differences after 10 minutes ($p = 0.030$) and greater differences after 60 minutes ($p = 0.005$; Figure 1C). No difference was detected between male WT and MPS IIIA mice (Figure 1C).

At 8 months of age there was a trend for the female mice to be hyperactive but no significant differences were found after 3 ($p = 0.73$), 10 ($p = 0.15$) or 60 minutes ($p = 0.18$; Figure 1D). Similarly, no significant differences were found with male mice after 3, 10 or 60 minutes. Overall, using MANOVA repeated measure analysis, a significant genotype difference ($p = 0.011$), time difference ($p = 1.2 \times 10^{-7}$) and a time*genotype difference ($p = 0.021$) were found. This indicates that overall, MPS IIIA mice are significantly more hyperactive than WT mice, that this changes with time and that MPS IIIA and WT mice change their behaviour in a different manner over time.

The number of unsupported rears (front paws off the floor), supported rears (front paws on a wall) and total rears were counted manually in the first 10 minutes of the open field test. The only significant change observed was a decrease in the number of unsupported rears by female MPS IIIA mice at 6 months of age (Figure 1F). However, given that there were no other significant genotype effects, this may be just a chance occurrence (Figure 1E and G). MANOVA repeated measure analysis of the data showed no significant genotype effect in the three rearing measures, but there was a significant decline in all measures with time (unsupported; $p = 0.004$, supported and total $p = 0.00003$) irrespective of genotype.

Rapid exploratory motion was analysed by measuring the frequency and duration of speed over 100 mm/s (Figure 2A–F). At 4 months of age, female MPS IIIA mice show significant increases in both the frequency ($p = 0.048$; Figure 2A) and duration ($p = 0.05$; Figure 2D) of rapid exploration over 60 minutes. However, no significant differences were observed after 3 or 10 minutes, although a trend was detected after 10 minutes (Figure 2A). No significant differences were found between male MPS IIIA or WT mice at 4 months of age.

At 6 months of age, there was a trend towards increased frequency (Figure 2B) and duration (Figure 2E) of rapid exploratory motion after 3 minutes in the female MPS IIIA mice ($p = 0.23$, $p = 0.10$). After 10 ($p = 0.021$, $p = 0.034$) and 60 minutes ($p = 0.0003$, $p = 0.0057$), female MPS IIIA mice had significantly increased frequency (Figure 2B) and duration (Figure 2E) of rapid exploratory motion over 100 mm/s. No significant differences were observed between male WT and MPS IIIA mice. This rapid exploratory behaviour is visible in Video S1 which shows the median female WT and MPS IIIA mouse at 6 months of age at 4 times normal speed.

At 8 months of age, female MPS IIIA mice show a trend towards increased frequency of rapid exploratory motion over 60 minutes ($p = 0.18$) (Figure 2C), with a trend to increase in duration after 10 minutes ($p = 0.083$) and a significant increase after 60 minutes ($p = 0.02$) (Figure 2F). Male MPS IIIA and WT mice were indistinguishable. Using MANOVA repeated measure analysis, there was a significant difference between WT and MPS IIIA mice over all three time points for frequency (Genotype; $p = 0.004$) and duration (Genotype; $p = 0.013$) of rapid exploratory motion. There was a significant decline in frequency (Time; $p = 9.7 \times 10^{-15}$) and duration (Time, $p = 4.5 \times 10^{-8}$) of rapid exploratory motion with time and also between genotypes over

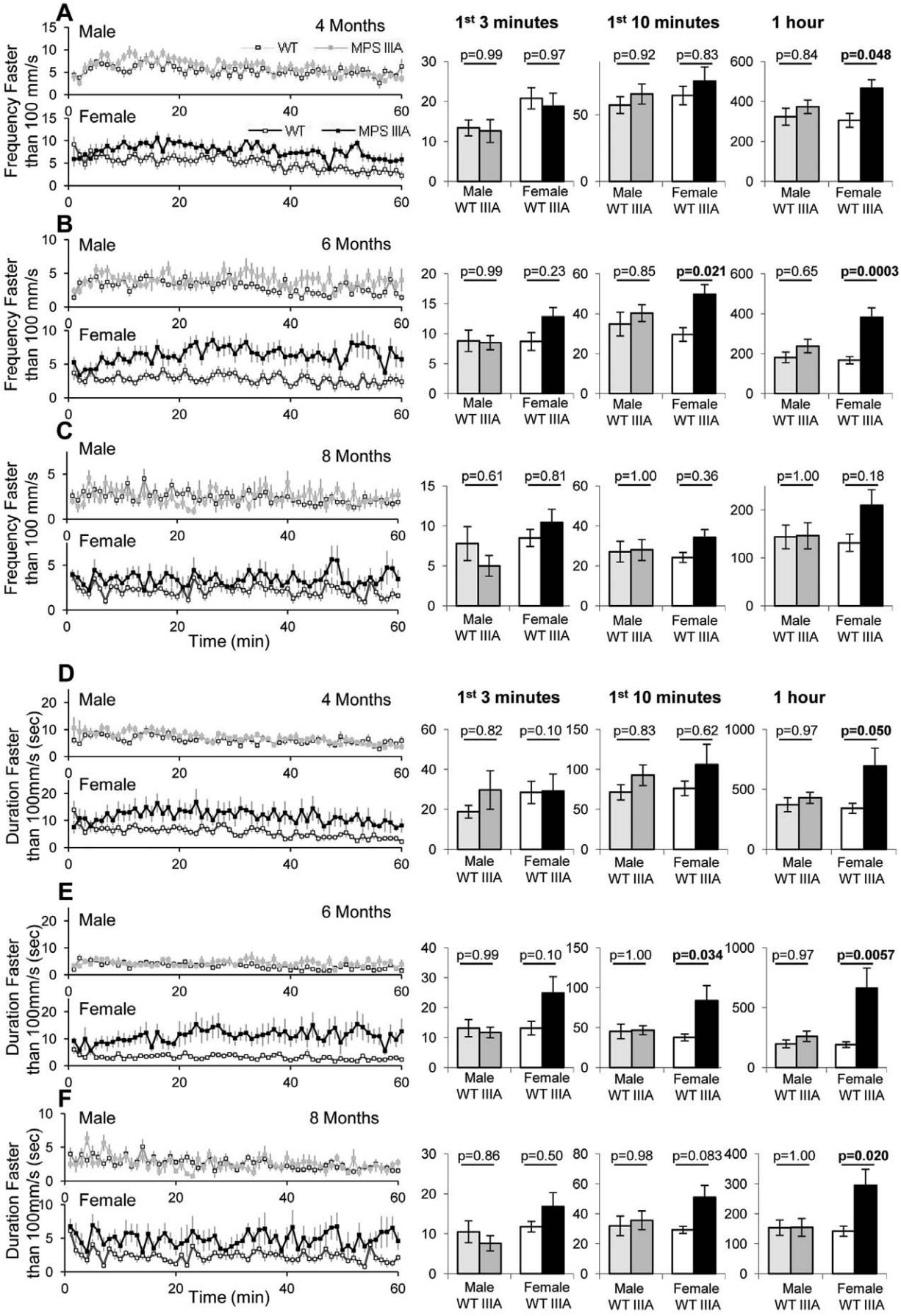
A
Male
4 Months
WT
MPS IIIA
Female
Frequency Faster than 100 mm/s
1st 3 minutes
1st 10 minutes
1 hour
p=0.99
p=0.97
p=0.92
p=0.83
p=0.84
p=0.048
B
6 Months
p=0.99
p=0.23
p=0.85
p=0.021
p=0.65
p=0.0003
C
8 Months
p=0.61
p=0.81
p=1.00
p=0.36
p=1.00
p=0.18
Time (min)
D
4 Months
Duration Faster than 100mm/s (sec)
p=0.82
p=0.10
p=0.83
p=0.62
p=0.97
p=0.050
E
6 Months
p=0.99
p=0.10
p=1.00
p=0.034
p=0.97
p=0.0057
F
8 Months
p=0.86
p=0.50
p=0.98
p=0.083
p=1.00
p=0.020
Time (min)
Male
Female
WT
IIIA

Figure 2. Open field rapid exploratory behaviour. At 4, 6 and 8 months of age 10 WT male (light grey squares), 10 MPS IIIA male (dark grey squares), 10 WT female (white squares) and 11 MPS IIIA female (black squares) were placed in the open field and the behaviour was recorded for 60 minutes. The results of this test are presented as a 60 minute period with the average of every minute presented and as a bar chart of the first 3 minutes, first 10 minutes and the whole hour. Error bars represent the SEM. p values were calculated by 2 Way ANOVA. The frequency of moving faster than 100 mm/s at 4 (A), 6 (B) and 8 (C) months and the duration spent moving faster than 100 mm/s at 4 (D), 6 (E) and 8 (F) months of age have been presented.
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time for frequency (Time*Genotype $p = 0.00005$) and duration (Time*Genotype, $p = 0.036$).

The frequency and duration the mice spent immobile was also recorded (Figure 3A–F). There were no significant differences in frequency of immobility between either male or female MPS IIIA and WT mice at any time point. However, female WT mice had significantly increased duration of immobility over 60 minutes at 4 ($p = 0.005$) and 6 months ($p = 0.004$) but not at 8 months ($p = 0.70$). Male MPS IIIA or WT mice had indistinguishable duration of immobility at all time points measured. Using MANOVA repeated measures there was an overall increase in the frequency of WT immobility over MPS IIIA mice over the three time points (Genotype; $p = 0.027$), but no change with time (Time; $p = 0.543$), or genotype with time interaction (Genotype*-Time; $p = 0.636$). MANOVA repeated measures analysis of duration of immobility demonstrated a significant genotype effect over the three time points (Genotype; $p = 0.05$), that changes significantly with time (Time; $p = 1.5 \times 10^{-9}$) and changes differently with time between the two genotypes (Genotype*Time; $p = 0.030$).

Mice being a prey species tend to display thigmotaxis, remaining close to the sides of an open field arena. Increased frequency and duration of time spent in the centre of the open field test therefore demonstrates reduced thigmotaxis and this is commonly considered to be a measure of reduced anxiety or reduced sense of danger that the animal experiences [37]. Male MPS IIIA and WT mice showed no significant differences in frequency of centre entries at any age (Figure 4A, B and C). At 4 months of age, female MPS IIIA mice showed significantly increased centre entries at 60 minutes ($p = 0.0045$). At 6 months female MPS IIIA mice entered the centre significantly more at 3, 10 and 60 minutes ($p = 0.057$, $p = 0.023$, $p = 0.0005$; Figure 4B). At 8 months of age female MPS IIIA showed no significant increases in centre entries ($p = 0.37$)(Figure 4C). Using MANOVA MPS IIIA mice showed significantly increased centre entries (Genotype; $p = 0.003$), which changed significantly over time (Time; $p = 2.3 \times 10^{-11}$) and between genotypes over time (Genotype*Time; $p = 0.0013$).

The duration spent in the centre has also been analysed. At 4 months of age, female MPS IIIA mice spent significantly more time in the centre than WT, after 60 minutes ($p = 0.012$; Figure 4D). Males were not significantly different. At 6 months of age female MPS IIIA mice had increase duration in the centre after 60 minutes ($p = 0.012$) and showed an almost significant trend in male mice ($p = 0.055$; Figure 4E). At 8 months of age there are no significant differences between genotypes (Figure 4F). By MANOVA repeated measure analysis MPS IIIA mice spend significantly increased duration in the centre area (Genotype; $p = 0.001$), this changes significantly over time (Time; $p = 2.5 \times 10^{-6}$) and between genotypes over time (Time*Genotype; $p = 0.005$).

Thirty minutes after behavioural analysis in the open field, the same cohort of mice were tested on an elevated plus maze (Figure 5A) for ten minutes, followed by a 30 minute rest and another 10 minute trial. Several parameters were measured (frequency of entering open arm, percentage of entries into the open arm, path length in open arm, percentage of path length in open arm and time in open arm) but none reached significance. Here we have presented the percentage of entries to the open arms, percentage of path length in the open arms and duration in the open arms as measure of anxiety (Figure 5B, C, D), which is representative of many of the other measures. At 4, 6 and 8 months of age there was no significant difference in male or female WT or MPS IIIA open arm entries (Figure 5B, C, D). At 4, 6 and 8 months there was no difference between WT and MPS IIIA mice in the repeat elevated plus maze and no difference between first and second elevated plus maze tests except that in the second test, all the mice performed fewer entries and had a shorter path length (Figure S1A–C). Statistical analysis by MANOVA showed no significant difference in genotype, or time but there was a significant difference in how the genotypes behaved over time in the percentage of path length in the open arm ($p = 0.019$) as the MPS IIIA mouse path length decreased at 8 months but the WT increased.

The inverted screen test (Figure 6A) and horizontal bar crossing test (Figure 6B) were also performed. The inverted screen test measures neuromuscular strength and the bar crossing test measures both neuromuscular strength and motor coordination. The bar crossing test showed no significant differences between WT and MPS IIIA of either sex at 4, 6 or 8 months of age (Figure 6C–E). Over time, there was a significant decrease in the number of moves and time spent on the inverted screen ($p = 1.7 \times 10^{-6}$, $p = 1.2 \times 10^{-5}$), and a significant sex difference in the number of moves with females moving more ($p = 0.004$). Bar crossing showed no genotype effect and no time effect but there was a significant Time*Sex effect ($p = 0.044$), as the female score decreased over time but the male score increased at 6 months and then decreased at 8 months.

Discussion

We have backcrossed the mixed background MPS IIIA mouse model onto the C57BL/6J background and characterised a behavioural phenotype that can be used to accurately distinguish female MPS IIIA mice from WT littermates. Our findings clearly show that male mice show no significant difference to WT littermates in the open field test or elevated plus maze, whilst female MPS IIIA mice demonstrate characteristic hyperactive behaviour initially at 4 months (16 weeks) and more strongly at 6 months (24 weeks), which declines again at 8 months (32 weeks) of age. We also show that these mice have a reduced thigmotaxis at 4 and 6 months of age which would indicate a reduced sense of danger. Hyperactivity and a reduced sense of danger are observed in the behavioural phenotype of the MPS IIIA patients [1,7,10,11].

In this study we observed no significant behavioural differences between WT and MPS IIIA male mice. This is in contrast to Hemsley *et al.* 2005 where hyperactivity was initially observed at 3 weeks, hypoactivity at 6 and 15 weeks and no changes at 10, 20 and 40 weeks in male mixed background mice [32]. Lau *et al.* 2008 used male MPS IIIA mice that had been back crossed onto the C57BL/6 background and observed no differences at 3, 5 and 15

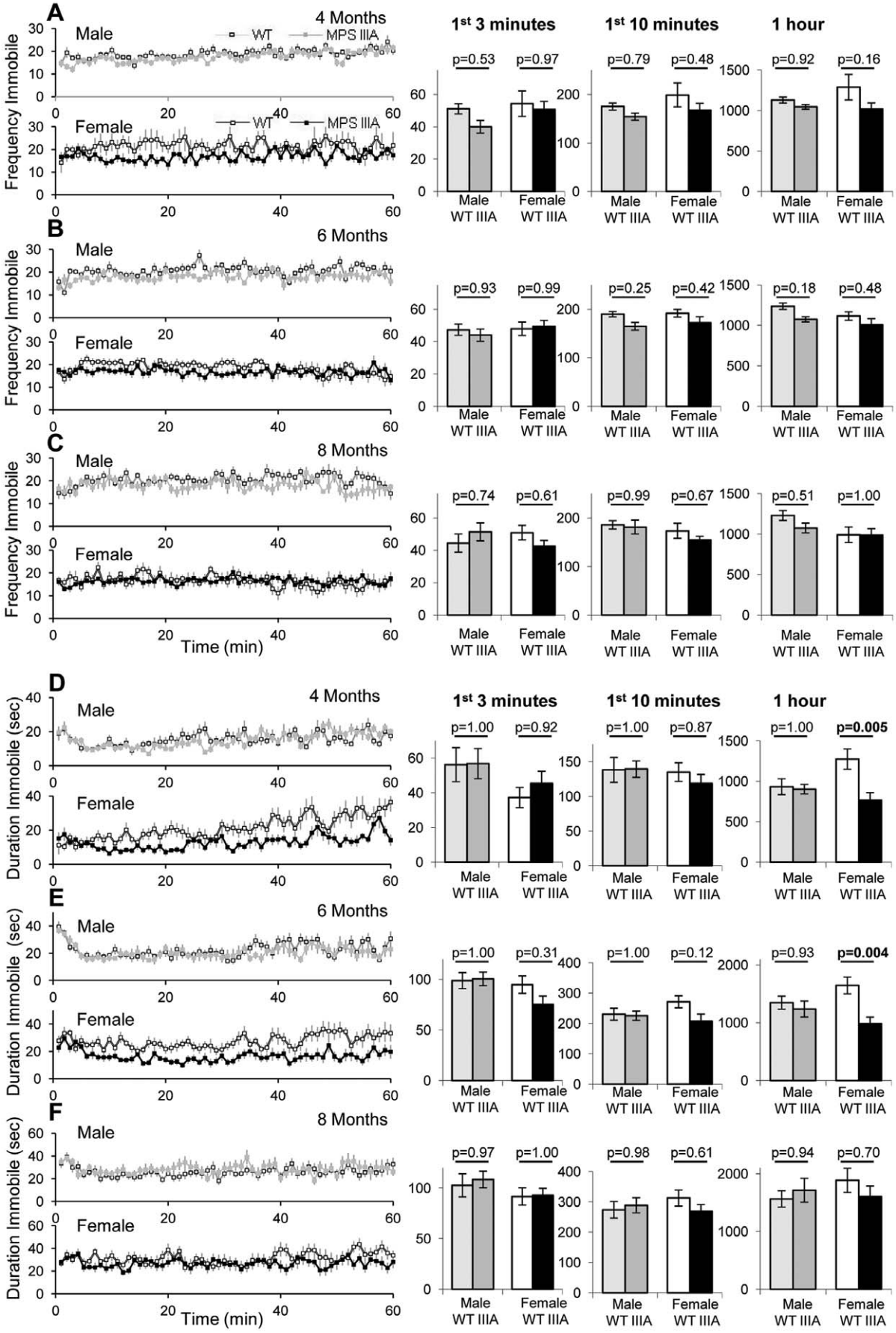
A
4 Months
Male
WT
MPS IIIA
Female
Frequency Immobile
B
6 Months
C
8 Months
Time (min)
1st 3 minutes
1st 10 minutes
1 hour
p=0.53
p=0.97
p=0.79
p=0.48
p=0.92
p=0.16
p=0.93
p=0.99
p=0.25
p=0.42
p=0.18
p=0.48
p=0.74
p=0.61
p=0.99
p=0.67
p=0.51
p=1.00
Male
WT IIIA
Female
WT IIIA
D
E
F
Duration Immobile (sec)
p=1.00
p=0.92
p=1.00
p=0.87
p=1.00
p=0.005
p=1.00
p=0.31
p=1.00
p=0.12
p=0.93
p=0.004
p=0.97
p=1.00
p=0.98
p=0.61
p=0.94
p=0.70

Figure 3. Open field immobile behaviour. At 4, 6 and 8 months of age 10 WT male (light grey squares), 10 MPS IIIA male (dark grey squares), 10 WT female (white squares) and 11 MPS IIIA female (black squares) were placed in the open field and the behaviour was recorded for 60 minutes. The results of this test are presented as a 60 minute period with the average of every minute presented and as a bar chart of the first 3 minutes, first 10 minutes and the whole hour. Error bars represent the SEM. p values were calculated by 2 Way ANOVA. The frequency of not moving at 4 (A), 6 (B) and 8 (C) months and the duration spent not moving at 4 (D), 6 (E) and 8 (F) months of age have been presented.
doi:10.1371/journal.pone.0025717.g003

weeks, with significant hypoactivity at 10, 18 and 22 weeks [33] but subsequently observed hypoactivity in 15 week old male mice [22]. Crawley *et al.*, using male MPS IIIA mice on the same C57BL/6 background, found no changes at 4, 6, 8, 10, 15, 18, 22, 25, 32 and 40 weeks and only observed hyperactivity at 12 weeks [30]. Finally, using male mice independently crossed onto the C57BL/6J background by Jackson laboratories, hypoactivity was observed in MPS IIIA mice at 20 weeks of age by McIntyre *et al.* but no difference was found at 24 or 32 weeks [38]. Clearly, it is hard to obtain consistent behavioural readouts from male mice in this context and it is always difficult to know if hypoactivity could be confounded by any parameter affecting the mouse's physical ability to move. We find male MPS IIIA mice to be very aggressive and are unable to keep them group housed, which is likely to change their behaviour. Singly housing male C57BL/6 mice has been shown to increase locomotor activity and reduce anxiety [39] or alternatively in other researchers hands to have no effect [40]. At the very least, it certainly leads to weight gains, which will restrict movement, and this may confound reliable open field measures. Additionally we find that all MPS IIIA mice retain urine over time, with male mice retaining significantly more urine at 8 months of age and urine retention is the humane endpoint (Figure S2). Clearly urinary retention could be restricting movement which would confound behavioural measures. Lastly, it is worth pointing out that many male mouse studies are on mixed, C57BL/6 or C57BL/6J backgrounds, some of which have been performed in different laboratories, which could lead to inconsistencies in outcomes. Overall, we would argue that comparisons of male MPS IIIA mice with WT are not appropriate (or easy to perform) for determination of treatment responses.

Several studies analysed behaviour of male MPS IIIA mice following control intracranial injections. Fraldi *et al.* tested male mice and found that the MPS IIIA mice were hyperactive at 21 weeks of age but not prior to this, but these mice had received intracranial injections and this may have adversely affected the mouse's behaviour [23]. Other studies where intracranial injections have been given to MPS IIIA mice all used male mice and generally observed either no changes or hypoactivity [18,21,35]. It is worth considering that intracranial injections could have altered the natural behaviour of the mouse and this may be why hypoactivity is observed.

We found female MPS IIIA mice to be hyperactive, having a significantly increased path length, frequency and duration of rapid exploratory behaviour and reduced duration of immobility at 4 and 6 months of age, (16 and 24 weeks) whilst duration of rapid exploratory behaviour was significantly increased at 8 months (32 weeks). At 4 months of age differences are only significant after 60 minutes, but at 6 months they are all significant after both 10 and 60 minutes. This indicates that the 60 minute test is a more sensitive test to identify hyperactivity than the 10 or 3 minutes tests. No significant differences were observed over the first 3 minutes at any time point, thus we would suggest that studies such as that of Crawley *et al.* 2006 where this short test has been used are not likely to yield significant differences [30].

Our hyperactivity findings in females are supported by Crawley *et al*, where hyperactivity was observed at 22 and 32 weeks of age in a 3 minute test with female MPS IIIA C57BL/6 mice, however no difference was observed at any point prior to 18 weeks or at 25 or 40 weeks of age [30]. They also observed hypoactivity in females at 4 weeks of age but we do not have comparative data to comment on this. The longer testing time utilised in our study may have meant that we were better able to detect behavioural changes in the mice that were not detectable with the 3 minute test.

On the mixed background, female MPS IIIA mice were hyperactive at 3 weeks of age but at subsequent ages no differences were observed at 6, 10, 15, 20, 30 or 40 weeks of age [32]. In the work of Lau *et al.*, using the C57BL/6 backcross, no differences were observed between female WT and MPS IIIA C57BL/6 mice between 3–22 weeks of age [33].

MPS IIIB is a phenotypically indistinguishable disease to MPS IIIA. In the mouse model of MPS IIIB, hyperactivity has been observed in a 10 minute open field test [41] but hypoactivity in an 8 minute open field test performed half in the light half in the dark [42]. A 60 minute open field test did not report differences in path length in male or female mice [43], but a 60 minute open field test that we performed on female mice at the same circadian time as this study observed hyperactivity at 8 months of age [36], which is consistent with our findings here. We also observed significant increases in hyperactivity in male MPS IIIB mice at 8 months of age, although they were less significant differences than those observed in females [14]. The MPS IIIB mouse is a complete knockout and thus may be slightly more severe than the MPS IIIA mouse with ~3% residual enzyme activity. This may explain why we were able to detect significant genotype differences at 8 months of age in MPS IIIB in all parameters but only some in MPS IIIA. We believe that the 60 minute open field test is still measuring habituation behaviour due to the profiles of activity observed in both MPSIIIA mice in this paper and in MPSIIIB mice [36] over time. We found that differences in behaviour between MPS IIIB and WT mice [36] and MPS IIIA and WT mice (Figure 1B and C) became more pronounced from 3 to 10 to 60 minutes and diverged in the first few minutes from a similar initial response. When we compared MPS IIIB and WT mice over a 24 hour period following 24 hours of habituation [36] the differences were significantly muted and over 14 days there was no significant difference [44]. This suggests that a 60 minute open field test does not reflect home cage behaviour but instead probably measures extended habituation to a novel environment.

A significant decrease in the number of unsupported rears by female MPS IIIA mice was observed at 6 months of age, however, there was no difference in the overall number of rears, or supported rears, at this or any other time point. No consistent trend in the number of rears with female mice is apparent in the literature, Crawley *et al.* reported that female MPS IIIA mice reared less at 15 and 25 weeks of age but there were no significant differences at 9 other time points [30]. No significant differences in rearing were observed in Lau *et al.* who also used backcrossed mice [33], or Hemsley *et al* who used mixed background mice [32]. A more consistent trend is observed in male mice with MPS IIIA rearing less [18,30,32,33,35], but increased rearing has been observed at 3 weeks [32]. However, at most time points no significant difference is observed. Manual measurement of rears

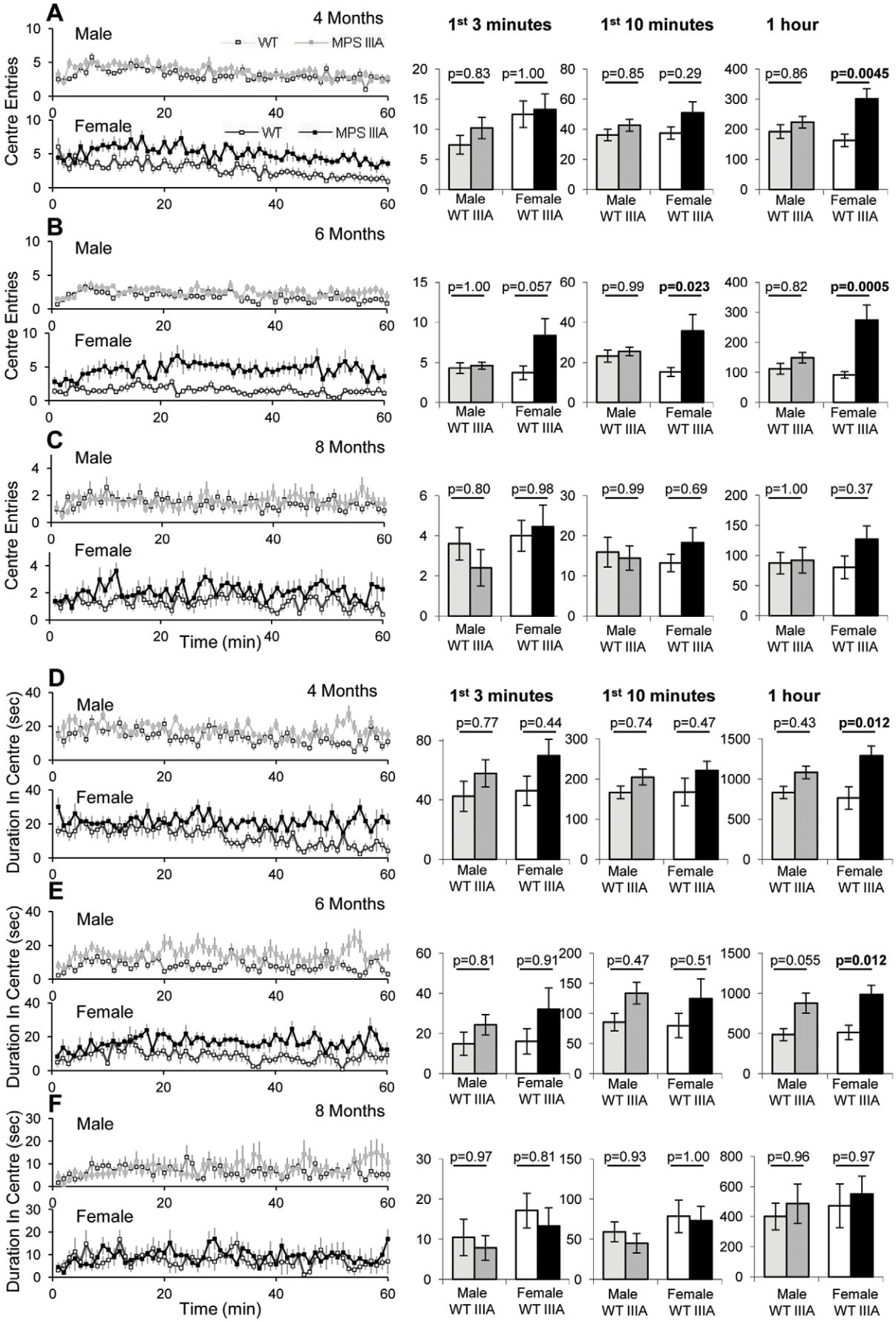
A
4 Months
Male
WT
MPS IIIA
Female
Centre Entries
B
6 Months
C
8 Months
Time (min)
D
Duration In Centre (sec)
E
F
1st 3 minutes
1st 10 minutes
1 hour
p=0.83
p=1.00
p=0.85
p=0.29
p=0.86
p=0.0045
p=1.00
p=0.057
p=0.99
p=0.023
p=0.82
p=0.0005
p=0.80
p=0.98
p=0.99
p=0.69
p=1.00
p=0.37
p=0.77
p=0.44
p=0.74
p=0.47
p=0.43
p=0.012
p=0.81
p=0.91
p=0.47
p=0.51
p=0.055
p=0.012
p=0.97
p=0.81
p=0.93
p=1.00
p=0.96
p=0.97
Male
WT IIIA
Female
WT IIIA

Figure 4. Open field sense of danger behaviour. At 4, 6 and 8 months of age 10 WT male (light grey squares), 10 MPS IIIA male (dark grey squares), 10 WT female (white squares) and 11 MPS IIIA female (black squares) were placed in the open field and the behaviour was recorded for 60 minutes. The results of this test are presented as a 60 minute period with the average of every minute presented and as a bar chart of the first 3 minutes, first 10 minutes and the whole hour. Error bars represent the SEM. p values were calculated by 2 Way ANOVA. The frequency of entering the centre at 4 (A), 6 (B) and 8 (C) months and the duration spent in the centre at 4 (D), 6 (E) and 8 (F) months of age have been presented.
doi:10.1371/journal.pone.0025717.g004

can be subjective and there may be variations in the rearing behaviour, such as amount of time per rear that cannot be measured by simply counting the number of rears. This is why we examined the number of supported and unsupported rears [36,45]. In the MPS IIIB mouse model, no difference in rearing was observed in the first 10 minutes of an open field test with female mice at 8 months of age [36] and no difference in the first 30 minutes but a significant decrease in the number of rears by 4.5–5 month old male MPS IIIB mice in the second 30 minutes of a 60 minute open field test [43]. Our conclusion is that rearing is too variable an outcome in MPS IIIA and IIIB mice and is therefore not a valuable informative test.

At 4 and 6 months of age the MPS IIIA female mice had a reduced sense of danger and spent a greater proportion of time in

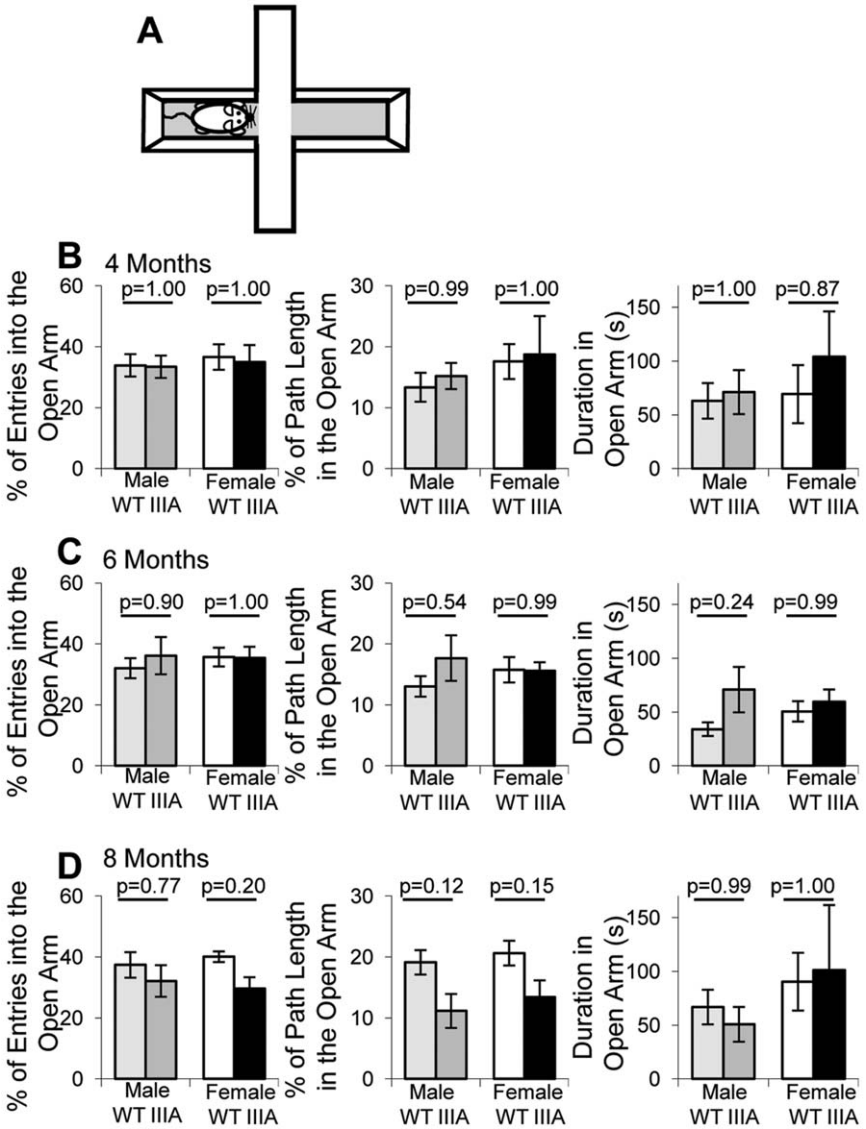

Figure 5. Elevated plus maze behaviour. At 4, 6 and 8 months of age 7–11 WT male (light grey bars), MPS IIIA male (dark grey bars), WT female (white bars) and MPS IIIA female (black bars) were placed on the elevated plus maze (A) and the behaviour was recorded for 10 minutes. After a 30 minute rest, the test was repeated. The results of the first test are presented as the mean of each measure with error bars representing the SEM. p values were calculated by 2 Way ANOVA. The following measures have been presented; the percentage of total entries that were into the open arm, the percentage of the path length in the open arm and the duration spent on the open arm at 4 (B), 6 (C) and 8 (D) months of age.
doi:10.1371/journal.pone.0025717.g005

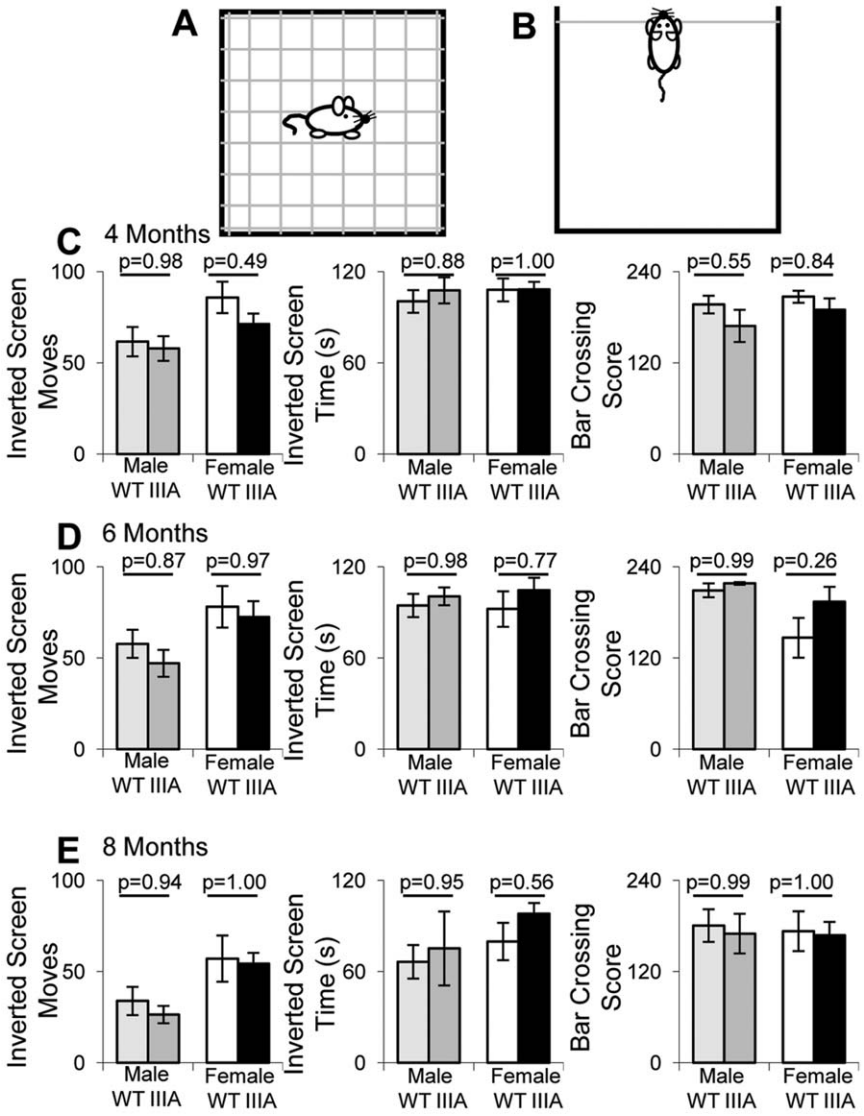

Figure 6. Neuromuscular behaviour. At 4, 6 and 8 months of age 8–11 WT male (light grey bars), MPS IIIA male (dark grey bars), WT female (white bars) and MPS IIIA female (black bars) were placed on the inverted screen test (A) and horizontal bar crossing test (B). The number of rear leg moves, the time spent on the inverted screen and the bar crossing score are presented at 4 (C), 6 (D) and 8 (E) moths of age. Error bars represent the SEM and p values were calculated by 2 Way ANOVA.
doi:10.1371/journal.pone.0025717.g006

the centre of the open field, this measure was significant after 60 minutes but not after 3 or 10 minutes. This is supported by the patients with MPS IIIA which are believed to have a reduced sense of danger [1]. No difference in the time in centre was detected by Lau *et al.* in both male and female mice [33], however this could be because the open field was larger in our study and therefore more sensitive to thigmotaxis [45,46]. In the MPS IIIB mouse model no differences were observed in the duration spent in the centre of the open field [36] but the open field used in that study was also smaller than the one used in this study.

The elevated plus maze did not demonstrate a decrease in anxiety or sense of danger in the MPS IIIA mice. There were also no differences between repeat tests, and both WT and MPS IIIA mice appeared to habituate equally. Lau *et al.* 2010, observed no significant differences with male mice at 6.5 months (26 weeks) of age with a 5 minute elevated plus maze test, but there was a trend towards reduced anxiety [34]. However in Lau *et al.* 2008 a significant increase in the time the male MPS IIIA mice spent in the open arms at 18 weeks of age, the percentage of the path length on the open arms at 15 and 18 weeks and the percentage of entries into the open arms at 20 weeks was observed [33]. Additionally they observed significant differences between repeats of the elevated plus maze, male MPS IIIA mice had a significantly longer path length in the repeat elevated plus maze test while WT mice remained unchanged [33]. We did not observe decreased anxiety or a change in behaviour between repeat elevated plus maze tests. The width of the arms could be affecting this test as this study used 10 cm wide arms and Lau *et al.*, used 7 cm wide arms. It has been demonstrated that when using 5, 7 or 9 cm wide arms, NMRI mice spent more time in the wider open arms compared to C57BL/10J mice that showed no difference [47]. Ten minutes on the elevated plus maze may also be too short a time to detect the sense of danger differences in the MPS IIIA mice as the only centre measure on open field that is significant after 10 minutes is

the number of centre entries in female mice at 6 months of age ($p = 0.023$). A lack of difference in the elevated plus maze performed in daylight is supported by MPS IIIB mice which showed no difference in the light but were less anxious in the dark [41]. The increased number of centre entries and duration in the open field could perhaps reflect increased but undirected anxiety whereby mice are actually more agitated but have lost the ability to determine what is dangerous. A more pragmatic explanation could be due to the mice not being naively tested as they only received a 30 minute break after the open field test before starting the elevated plus maze test. Prior behavioural tests can affect the result of elevated plus maze tests with C57BL/6J mice that have previously been tested in an open field test [48].

No significant differences were observed in the inverted screen or bar crossing tests which is consistent with the work of Crawley *et al.* who observed no difference in neuromuscular strength with back crossed MPS IIIA mice [30]. However on the mixed background strain, there was a significant decrease in neuromuscular strength with MPS IIIA mice being unable to grip the inverted screen for as long as the WT mice from 20 weeks of age [32]. The work in our study is supported by work with the MPS IIIB mouse model that does not show a decline in motor function at 5 months of age by accelerating rotarod [43,49] but does by a rocking rotarod test from 9 months onwards [50,51]. The decline in motor function could relate to the presence of increasing urine retention in the MPS IIIA and B mouse models [14,26,29]. Our own tests in the MPS IIIB mouse model found that neuromuscular decline only occurred at 10 months of age (40 weeks) [14,36]. Given that the MPS IIIA mouse has residual enzyme activity, it is possible that progression of MPS IIIA disease is slightly less rapid than in MPS IIIB which would explain why we did not see a trend to changes in motor function at 8 months in MPS IIIA mice.

Standardisation of tests is a very important aspect of behavioural test design that may have affected comparison of our tests to those of other laboratories. In this study the size of the open field was larger than that described in many studies and the behaviour was observed for much longer (60 minutes compared to 3 minutes). The increased size and time of the test allows better spatial and temporal resolution of the mouse behaviour and so makes the test more sensitive [45]. Video analysis software was used which has been shown to be more sensitive than line crossing determined by an observer or beam breaks [52]. In this study, the mouse was placed in the centre of the open field rather than in the corner and the test was carried out at the same time point of circadian rhythm for all mice as there is a known peak in activity in the similar MPS IIIB mouse model [44]. The mice in this study were also housed differently; females were group housed and male mice were singly housed from 14 weeks of age. The background of the mice can affect the behavioural phenotype, therefore we backcrossed the MPS IIIA mice in this study onto the C57BL/6J background for more than 10 generations. Other groups have used MPS IIIA mice on a mixed background [18,32], or on a C57BL/6 [23,30,33,34] or C57BL/6J [21] background. Strain specific differences can also significantly affect behavioural outcomes [53].

Conclusion

We have demonstrated that we can reliably detect differences in the behaviour of female but not male MPS IIIA mice at 4 and 6 months (16 and 24 weeks) of age and these differences match the patient phenotype. Male mice were singly housed due to their aggression which may change comparative behavioural responses. Female MPS IIIA mice are hyperactive, with a longer path length, increased frequency and duration of rapid exploratory behaviour, and spend less time immobile. They also show a trend to this behaviour at 8 months of age with significantly increased duration of rapid exploratory behaviour. Female MPS IIIA mice demonstrate a reduced sense of danger with a greater proportion of the time spent in the centre of the open field with a greater number of centre entries. These two time points and multiple measures are ideal to evaluate novel therapies for MPS IIIA as the effect of a therapy can be sensitively determined using multiple measures. This behavioural phenotype is supported by both the phenotype of the patients and our recent similar observations in the MPS IIIB mouse model [14,36]. The increased size of the open field, the increased length of the test, the use of video analysis software and performing the test at consistent point in the circadian rhythm have produced a sensitive and robust test to evaluate the effect of therapies on female MPS IIIA mice.

Supporting Information

Figure S1 Repeat elevated plus maze behaviour. At 4, 6 and 8 months of age 7–11 WT male (light grey bars), MPS IIIA male (dark grey bars), WT female (white bars) and MPS IIIA female (black bars) were placed on the elevated plus maze (A) and the behaviour was recorded for 10 minutes. After a 30 minute rest, the test was repeated. The results of the repeat test are presented as the mean of each measure with error bars representing the SEM. p values were calculated by 2 Way ANOVA. The following measures have been presented; the percentage of total entries that were into the open arm, the percentage of the path length in the open arm and the duration spent on the open arm at 4 (A), 6 (B) and 8 (C) months of age.
(TIF)

Figure S2 Urine retention at 8 months. At 8 months of age 9 WT male (light grey bars), 8 MPS IIIA male (dark grey bars), 7 WT female (white bars) and 8 MPS IIIA female (black bars) were sacrificed and the urine volume in the bladder measured. The results are presented as the mean with error bars representing the SEM. p values were calculated by 2 Way ANOVA.
(TIF)

Video S1 Open field behaviour video. A video running at 4 times the normal speed showing the median female WT and MPS IIIA mouse at 6 months of age. The MPS IIIA mouse is on the left and the WT mouse is on the right.
(WMV)

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Author Contributions

Conceived and designed the experiments: AL-S KJL-S FLW BWB. Performed the experiments: AL-S KJL-S. Analyzed the data: AL-S KJL-S FLW BWB. Wrote the paper: AL-S KJL-S SAJ RFW JEW FLW BWB.

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